ISSN 0973-3930

Volume 38 | Issue 1 | January–March 2018

International Journal of Diabetes in Developing Countries

Official Publication of
Research Society for the
Study of Diabetes in India

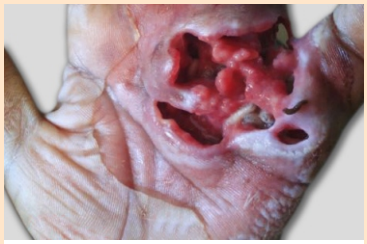
Springer

In Chronic Wound Management

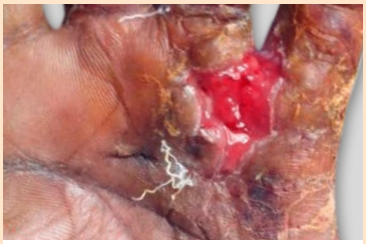
ZiFi TURBO

LINEZOLID 600MG + CEFIXIME 200MG **Tablets**

A Case of Chronic Wound



Before Treatment



After Treatment

Case : 60 year diabetic male with H/o Chronic Wound having Maggots.

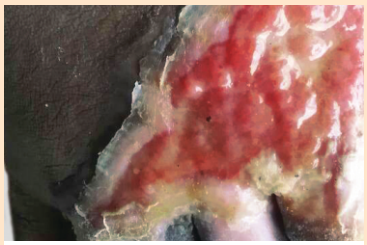
Treatment : ZiFi TURBO b.i.d. for 15 days.

In Acute Wound Management

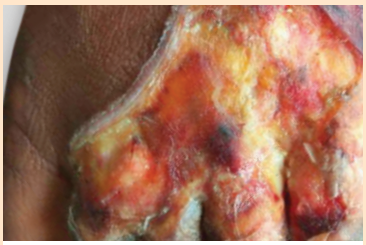
ZiFi TURBO 600

LINEZOLID 600MG **Tablets**

A Case of Acute Wound



Before Treatment



After Treatment

Case : 28 year old male with H/o **Electric Thermal Burn Injury**

Treatment : ZiFi TURBO 600 b.i.d. for 21 days

FDC SPECTRA **FDC Limited** 142-48, S.V. Road, Jogeshwari (West), Mumbai 400 102.

International Journal of Diabetes in Developing Countries
Incorporating Diabetes Bulletin

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International Journal of Diabetes in Developing Countries targets a readership consisting of clinicians, research workers, paramedical personnel, nutritionists and health care personnel working in the field of diabetes. Original research work and reviews of interest to the above group of readers is considered for publication in the journal.

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Subscription Information

International Journal of Diabetes in Developing Countries is published 4 times a year. Volume 38 (4 issues) of will be published in 2018.

ISSN 0973-3930 print version
ISSN 1998-3832 electronic version

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International Journal of Diabetes in Developing Countries

Volume 38 · Number 1 · January–March 2018

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International Journal of Diabetes in Developing Countries (January–March 2018) 38(1):1–3
https://doi.org/10.1007/s13410-018-0614-5

EDITORIAL

Diabetes in pregnancy—a critical window of opportunity

S. V. Madhu[1]

Published online: 10 February 2018

Gestational diabetes mellitus (GDM) is a public health priority in our country due to its high prevalence as well as its immense potential for diabetes prevention. The realization that diabetes in pregnancy is a significant contributor to the growing epidemic of type 2 diabetes mellitus (T2DM) has also helped focus our attention on the pregnant woman as a critical target for diabetes prevention strategies.

Diabetes in pregnancy has serious consequences for the mother as well as the baby. The HAPO study found increasing incidence of these complications with rising maternal glucose [1]. In the mother, complications include still birth and a greater need for cesarean section and in the babies, it can cause large babies and congenital malformations. These can affect outcome of pregnancy and it is essential that diabetes is detected early and managed appropriately so that these consequences can be prevented.

The clinical issues that follow GDM in pregnancy are well appreciated. However, the transformation of GDM as a major public health issue is because it may play a crucial role in the increasing prevalence of diabetes and obesity [2]. In fact, GDM is believed to be a stage in the evolution of type 2 DM [3].

Hyperglycemia in pregnancy has its highest prevalence in South-East Asia, where one in four pregnancies is affected. Asians develop GDM at a lower BMI and type 2 DM occurs at a much younger age. With urbanization, GDM prevalence is becoming an epidemic [4]. In India, the prevalence of GDM varies from 3.8 to 21% in different parts of the country [5]. Approximately 7% of all pregnancies are complicated by GDM, resulting in more than 200,000 cases annually [6]. Indian women have 11-fold increased risk of developing glucose intolerance during pregnancy compared to Caucasian women [7].

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GDM is also a known risk factor for T2DM [8] besides its known adverse impact on pregnancy outcome. Women with GDM have a seven fold higher risk of developing T2DM. This risk increases steeply 5 years after delivery [9, 10]. Women with a history of GDM also have a higher prevalence of metabolic syndrome and increased risk of cardiovascular disease (CVD) [11].

Children of GDM mothers are at a higher risk of developing T2DM later in life [21 vs 4%] compared with children of non GDM mothers [12]. Babies born to mothers with gestational diabetes also have a higher lifetime risk of obesity and developing type 2 diabetes. GDM may be responsible for 19–30% of diabetes in some populations [13]. About one third of children born of diabetes pregnancies develop glucose intolerance before the age of 17 years [14].

Direct evidence of the benefits of interventions in the prevention of future of T2DM in the context of GDM also exists. Postpartum lifestyle intervention prevents type 2 DM and cardiovascular disease in women with GDM. Intensive lifestyle and metformin are highly effective in delaying or preventing diabetes in women with IGT and a history of GDM [15, 16]. More importantly, the pregnant mother with diabetes is a very effective starting point for a diabetes prevention strategy as she is highly motivated and carries the message to the family effectively.

The pregnant diabetic mother provides a critical link for transgenerational transmission of diabetes which sets off a self-perpetuating cycle of rising diabetes in the community. The hyperglycemia associated with GDM results in fetal overnutrition and a higher risk of obesity and diabetes in the offspring through a variety of mechanisms. These include epigenetic changes in the exposed offspring [17].When postnatal overnutrition gets added to this scenario, there is higher childhood and adolescent obesity and early onset adult type 2 diabetes. This in turn increases the prevalence of GDM and sets off a vicious cycle of GDM and T2DM in the community [18]. Epigenetic changes in various genes may increase the lifelong metabolic disease susceptibility and, thus, the likelihood for a new generation of mothers with GDM and/or obesity thus feeding the vicious cycle [17]. Preventive measures against

type 2 DM should start during intra uterine period and continue throughout life from early childhood [19].

Asian Indians are a high-risk ethnic group for development of diabetes. Hence, all pregnant Indian women need to be screened for GDM. There is an urgent need to screen all mothers for diabetes early enough to detect and initiate appropriate treatment to prevent and minimize its effects not only on mother and the fetus but also to prevent the transgenerational propagation of diabetes and contain the diabetic epidemic in the community.

There are two articles in this issue which focus on early detection and optimizing treatment in GDM. While the study by Melekoglu et al. [20] evaluates specific screening strategies to enhance greater detection of GDM in the first trimester, the studies by Xia et al. [21] have evaluated the effects of lifestyle in the control of glycemia in GDM. Using the criteria of the International Association of Diabetes and Pregnancy Study Groups (IADPSG), Melekoglu et al. [20] showed that 80% of GDM cases could be detected in the first trimester itself, thus offering the scope for early intervention. The meta-analysis by Xia et al. clearly confirms the beneficial effect of lifestyle interventions in controlling both fasting and postprandial hyperglycemia in GDM.

From the above, it is clear as to why the pregnant woman has emerged as an important target group for diabetes prevention. GDM offers an important opportunity for the development and implementation of various strategies for diabetes prevention [22]. Screening and identifying women with diabetes or GDM provides us an opportunity to make significant contributions which have great public health relevance. The high risk of gestational diabetes mellitus in pregnant women of our country and its early detection and effective treatment can prevent all adverse outcomes of pregnancy and result in a normal, healthy mother and baby after delivery. Diabetes in pregnancy is associated with a higher risk for future development of diabetes both for the mother and for the babies. Diagnosis of GDM helps us identify women at very high risk of future diabetes and CVD and by offering preventive care provides us the opportunity to prevent or delay diabetes and CVD and their complications. It also helps identify individuals—offsprings of GDM pregnancy, who are at considerable high risk of diabetes and other noncommunicable diseases (NCDs) and offer them early preventive care to contain the rising burden of T2DM and other NCDs.

Pregnancy offers a huge window of opportunity to not only improve pregnancy outcomes in GDM but also to address intergenerational prevention of NCDs, such as diabetes and cardiovascular disease.

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International Journal of Diabetes in Developing Countries (January–March 2018) 38(1):4–10
https://doi.org/10.1007/s13410-018-0605-6

POSITION STATEMENT

Diabetes and travel

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Published online: 1 February 2018

Summary

Travel exposes an individual in unfamiliar environments. Those planning to travel should schedule an appointment with their treating physician, at least a month in advance of their trip for an updated assessment of glycemic control, and should also procure a prescription describing the patient's medical condition, and medication. A diabetic individual should also carry extra amount of medicines which are distributed properly. Information regarding the climate and environmental conditions of the destination is a must. Extremes of weather can adversely affect the health of the patient and/or degrade medications, supplies, and equipment. Patients with diabetes are more susceptible to environmental stressors than their counterparts, such as increase incidence of heat exhaustion, cold exposure, or foot ulcers. Food options for diabetics may be limited during travel and travel planning should offer greater flexibility in dietary choices. Packing healthy snacks in carry-on luggage can take care of disrupted dietary patterns. Insulin concentration varies in various countries, and hence, the use of the correct syringes is essential. Unit of blood glucose measurement may also be different. Availability of medications may also be an issue. So, it is important to carry a list of all medications with generic name and their dosages. Immunization against common and travel-related vaccine-preventable diseases is recommended. Those on insulin pump therapy should get in touch with the manufacturing company and it is advisable to disconnect the pump during takeoff or landing as change in cabin pressure may lead to excess insulin delivery. Individuals with diabetes should also carry travel health insurance. One must carry physician prescription, health insurance policy, medications and prescriptions for them, rescue medications, snacks, supplies, glucometers, coolants, pumps in double, first aid kit, comfortable shoes, and protective clothing. Airport security requiring patients on pump or continuous glucose monitor (CGM) to go

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through scanners should be warned from doing so as it may cause radiation-induced malfunction. These devices should not be removed also. Air travel requires patients to carry carbohydrate snacks, insulin, insulin pump, and medications in carry-on baggages to maintain temperature stability. Due to pressure differences in the cabin area, there might be some irregularities in insulin administration. Even in insulin pumps, similar issues may arise due to bubbles precipitating out of insulin solution in the microtubules. Blood sugars must be frequently checked. Traveling across less than five time zones does not require insulin dose adjustments, but in greater than five time zones, dose and timings need to be adjusted. Those with diabetes are at an increased risk of developing deep venous thrombosis, so they should be encouraged to stand and walk during long flights every 1–2 h and perform seated dorsiflexion/ plantarflexion exercises to avoid venous stasis; also, one must remain well hydrated. Train travel is much more flexible; though health insurance is not required, one must pack the same essentials in carry-on bags. During travel, there will be an inadvertent increase in walking, for which insulin requirement will decrease, and frequent snacking may also help. Blood sugar must be measured more frequently. One must wear proper footwear to avoid ulcers and infections. One must be properly hydrated and must also avoid diarrhea.

Keywords Diabetes · Insulin concentration · Health insurance

Recommendations

1. A diabetic individual should undergo a proper medical evaluation at least a month in advance of their trip as an individual is exposed to an unfamiliar destination.
2. Information regarding climate and environmental conditions of the destination is a must as extremes of temperature may have a deleterious effect on patients and may degrade medicines.
3. Availability of medicines, unit of blood glucose measurement, and insulin concentrations may also be an issue.
4. Individuals must carry a list of all medications with generic name and their dosages; medicines should be carried in extra in easily accessible bags.
5. Health insurance is a must and patients should also be immunized with vaccine-preventable diseases.
6. Airport security requiring patients going through body scanners should be careful as pumps and CGM may undergo radiation-induced malfunction.
7. In air travel, patients should carry medicines and carbohydrate-rich snacks in their carry-on luggages.
8. In air travel, patients should not inject insulin or use a pump at takeoff or landing due to pressure differences which may lead to irregularities in insulin administration.
9. Traveling across more than five time zones requires insulin dose and frequency adjustment.
10. In air travel, there is an increased risk of developing deep venous thrombosis (DVT), which can be easily prevented by simple exercise and hydration.
11. Train travel is more flexible; health insurance is not required but snacks and medications should be carried in easily accessible bags.
12. During travel, there will be an inadvertent increase in activities, and hence, medications should be adjusted and blood sugar level should be checked regularly.

Diabetes and travel

Travel, whether for pleasure or business, places an individual in unfamiliar environments. Those with chronic illnesses, like diabetes, may be vulnerable to the emotional and physical stresses associated with traveling. Diabetes is largely self-managed; however, when unfamiliar foods, climate, time zone changes, and living conditions are considered during times of travel, patients may face challenges in managing their diabetes [1]. A study conducted in Aberdeen, UK, showed that 15% of insulin users stated that their use of insulin affected their choice of travel destination, both in terms of health risk in developing countries and avoidance of long-haul travel [2]. However, individuals with diabetes can travel safely with adequate preparation and appropriate self-management skills.

This document aims to present a summary of recommendations and clinical insights that can be provided to patients with diabetes who are preparing to travel.

Travel exposes an individual in unfamiliar environments.

Pretravel recommendations

- Visit treating Health Care Professionals (HCP): Patients with diabetes who are planning to travel should schedule an appointment with their treating physician, at least a month in advance of their trip to allow for planning of diabetes care when traveling. This will also allow an updated assessment of glycemic control, evaluation and review of the risks of travel, and a discussion of the measures the patient can take to minimize these risks. In addition, the physician can remind the patient and reiterate on some important self-management principles, e.g., recognition and treatment of hypoglycemia symptoms, sick day guidelines, and self-monitoring of blood glucose requirements.

It is important to procure a prescription/letter from the physician describing the patient's medical condition, their current diabetes medication regimen, and the patient's medical necessity to carry sharps, e.g., needles and lancets, if the patient is on an insulin regime [3, 4].

It is prudent to advise the patients to plan for travel delays and lost luggage, so taking twice as many diabetes supplies and medications is recommended, preferably distributed in different luggage bags.

Those planning to travel should schedule an appointment with their treating physician, at least a month in advance of their trip for an updated assessment of glycemic control, and should also procure a prescription describing the patient's medical condition, and medication. A diabetic individual should also carry extra amount of medicines which are distributed properly.

- Knowing the destination: It is of utmost importance to research information regarding the climate and environmental conditions of the destination. Extremes of weather can adversely affect the health of the patient and/or degrade medications, supplies, and equipment [5, 6].

  Patients with diabetes are more susceptible to environmental stressors than their counterparts.

  Patients taking insulin or other injectable medications that are temperature sensitive should investigate the availability of refrigeration, e.g., refrigerators in hotel rooms and travel cold packs, at their destination and plan if such facilities do not exist, i.e., travel cold packs can be packed prior to departure.

  Suitable clothing should be carried based on the climate at the destination. Protective gear such as hats/sunglasses/sunscreen, gloves/mittens/boots, and comfortable footwear will enable patients with diabetes to enjoy their trip without putting themselves at higher risk for heat exhaustion, cold exposure, or foot ulcers [7].

Information regarding the climate and environmental conditions of the destination is a must. Extremes of weather can adversely affect the health of the patient and/or degrade medications, supplies, and equipment. Patients with diabetes are more susceptible to environmental stressors than their counterparts, such as increase incidence of heat exhaustion, cold exposure, or foot ulcers.

- Diet: Food options for patients with diabetes may be limited during travel, especially if one is traveling out of the country, so planning in advance is important. This is more relevant during air travel, as travel by road/train and maritime travel offer greater flexibility in dietary choices [8]. For flights during which a meal will be served, there is an option of selecting your choice of meal well in advance. The destination and flight duration are also important with regard to food options available. Packing healthy snacks in carry-on luggage can take care of disrupted dietary patterns that may occur during the flight. Access to such foods may be limited during travel and it is recommended to be carried to help prevent or treat hypoglycemic events. When traveling to countries where English is not the primary language, food labels and restaurant menus may be difficult to interpret. In such situations, investigating specific dietary options before departure, via the internet, may be helpful. When unsure, it is best to rely on known low-carbohydrate options, e.g., salads, nuts, and eggs [9].

Food options for diabetics may be limited during travel and travel planning should offer greater flexibility in dietary choices; *packing healthy snacks in carry-on luggage can take care of disrupted dietary patterns.*

- Medication: For insulin users, it is important to note that insulin concentration varies in various countries. Available options include U-40, U-100, or U-200. Use of the correct syringes with specific insulin concentrations is essential, since using wrong syringes may deliver incorrect dose of insulin. This concern is diminished in those who use insulin pens rather than vial and syringes.

  It is also important to note that the unit of blood glucose measurement in India is mg/dL, but many other countries use mmol/L. This will be important if someone's glucose meter malfunctions while abroad and another one needs to be bought locally.

  Travelers should be also aware that not all insulins, other injectables, or oral diabetes medications available in India will be available in every country throughout the world and that medications may be referred to by different names [10]. Therefore, it is important to carry a list of all medications with generic name and their dosages.

  Those who are on insulin pump therapy should get in touch with the manufacturing company for contact details at the destination, should there be a need. They must discuss with their treating physician about an alternative basal-bolus insulin regimen in the event of pump failure.

  Immunization against common and travel-related vaccine-preventable diseases is recommended, as per individual country recommendations, prior to departure. *It is advisable to disconnect the pump during take-off or landing as change in cabin pressure may lead to excess insulin delivery.*

Insulin concentration varies in various countries, and hence, the use of the correct syringes is essential. Unit of

blood glucose measurement may also be different. Availability of medications may also be an issue. So, it is important to carry a list of all medications with generic name and their dosages. Immunization against common and travel-related vaccine-preventable diseases is recommended. Those on insulin pump therapy should get in touch with the manufacturing company and it is advisable to disconnect the pump during takeoff or landing as change in cabin pressure may lead to excess insulin delivery.

- Travel health insurance: Where feasible, travelers need to get in touch with their medical insurance companies and review their medical coverage policies during travel should unforeseen emergencies arise. One should have easy access to their health insurance identification card. It is also important to locate the nearest hospital and pharmacy at the destination, before arrival, in case medical assistance is required. It would be wise to ensure that the health insurance is accepted at these facilities beforehand to avoid expensive medical bills or unforeseen costs.

Individuals with diabetes should also carry travel health insurance.

What to pack [1]

- Physician prescription/letter with the following information:

– Letter should be in English.
– Whether the patient has type 1 or 2 diabetes.
– Medications (generic name) and dosages—if on insulin pump, settings and basal-bolus backup regimen, in case of pump malfunction, should be included.
– Rescue medications, viz glucose gel, tablets, and a glucagon pen.
– Supplies with quantities mentioned (glucometer, testing strips, lancets, syringes/pens, and batteries).
– Necessity to carry sharps (needles and lancets).
– Physician name and contact details.

- Health insurance policy/card or details
- Diabetes medications and prescriptions for them

– Always keep double medicines and supplies than needed for travel. Do not pack them all in one place. Keep half the supplies in a bag that will be with the concerned individual in person, irrespective of mode of travel.

- Rescue medications (glucose gel, tablets, and glucagon pen)
- Supplies (syringes, lancets, test strips, sharps container)
- Two glucose meters (in case one fails) with extra batteries
- If on insulin pump, twice as many pump supplies as may be needed
- Coolant/cold packs/insulin wallets for insulin users
- First aid kit
- Comfortable shoes
- Protective clothing, depending on destination climate
- Some snacks to avoid hypoglycemia

One must carry physician prescription, health insurance policy, medications and prescriptions for them, rescue medications, snacks, supplies, glucometers, coolants, pumps in double, first aid kit, comfortable shoes, and protective clothing

Air travel

- Airport security [1]: Travel security, both national and international, has become strict in recent years. When traveling by air, outside the country, passengers should contact the airline to find out if the destination country has any specific airport security restrictions or requirements regarding diabetes medications or equipment. If a traveler is on an insulin pump or a continuous glucose monitor (CGM), it is important to ensure that the device not be removed since it is attached via a catheter underneath the skin. It is also prudent to check with the pump CGM manufacturing companies regarding recommendations for radiation exposure. Several companies allow the passage of their equipment through metal detectors but do not recommend that their products should be run through the x-ray machines or body scanners that implement x-ray technology, due to the potential risk of radiation-induced malfunction. This information may be available online and can be printed and shown to security personnel.

Airport security requiring patients on pump or CGM to go through scanners should be warned from doing so as it may cause radiation-induced malfunction. These devices should not be removed also.

- Storing diabetes medications and supplies: Carrying snacks that contain carbohydrates is a good backup in addition to glucose tabs, gels, or glucagon kits, in case blood sugars fall low. Carrying diabetes supplies in carry-on luggage is also a beneficial for several reasons:

– Easier accessibility while traveling.
– Avoids medications and supplies getting lost in case of luggage loss.
– Temperature extremes occur more frequently in the luggage compartments rather than in the cabin areas on

airplanes, which is important to consider when carrying insulin vials or pens.

Injectable diabetes medications have optimal storage temperatures between 2 and 8 °C while oral medications can be stored between 20 and 30 °C [11].
Insulin pumps have temperature tolerances of 5–40 °C and CGM devices from 10 to 40 °C; but specific temperature ranges vary by manufacturer.
Blood glucose testing strips should be kept in their tightly sealed containers to avoid exposure to moisture. Do not expose them to extreme temperatures.
Travelers should read the package inserts of their medications, devices, and equipment to ensure proper functioning.

In India, very recent Clinicare (India) Pvt. Ltd., a Mumbai-based company, has launched the FRIO ® Insulin Wallet. This is meant for keeping insulin cool while traveling and is a good option when one has no access to refrigeration or during power shortages while traveling.

Unlike traditional insulin carrying cases, FRIO®'s cooling properties are not derived from an ice pack or anything that needs refrigeration. It is easily activated by water. It is an environment-friendly green reusable product and is convenient to be carried around on oneself or in one's hand baggage [12].

Air travel requires patients to carry carbohydrate snacks, insulin, insulin pump, and medications in carry-on baggages to maintain temperature stability.

- Insulin on board: Depending on the duration of the flight, insulin may need to be administered on board an airplane. Due to pressure differences in the cabin area, resistance may arise when utilizing syringe plungers to draw up insulin [13]. Similarly, with insulin pen devices, there may be a leak in insulin when applying the pen tip needle for use.

  For those using insulin pump therapy on board an aircraft, recent data suggests the possibility of unintended insulin delivery during ascent from bubbles precipitating out of insulin solution in the microtubules according to pressure gradients [14]. In addition, there have been reports of significant unintended insulin administration due to plunger movements during rapid cabin depressurization, during emergency. Overall, more data is needed before recommendations regarding insulin pump management during flight can be made [15]. Travelers must check their blood sugars frequently due to the effects that stress, altered eating habits, and altered medication administration times may have on overall blood glucose control.

Due to pressure differences in the cabin area, there might be some irregularities in insulin administration. Even in insulin pumps, similar issues may arise due to bubbles precipitating out of insulin solution in the microtubules. Blood sugars must be frequently checked.

- Traveling across time zones: Diabetes management is based on a 24-h cycle. When traveling from west to east, one should remember that the day shortens compared with when traveling from east to west, when the days become longer [16]. Usually, if fewer than five time zones are crossed during travel, adjustments to insulin dosing are generally not necessary [17]. If more than five time zones are crossed, specific recommendations should be made by the treating physician to discuss how insulin dosing or timing of administration should change based on time zone differences.

  For those on oral medications, timing is less important. Patients should be educated not to take their sulfonylurea if they will be missing meals during travel to avoid hypoglycemia. However, other oral agents may be continued.

  Generally, it is helpful if travelers keep their wrist watch set to their departure time zone at least for the first day of travel.

Traveling across less than five time zones does not require insulin dose adjustments, but in greater than five time zones, dose and timings need to be adjusted.

- Prevention of venous thromboembolism in air travelers: Those with diabetes may be at an increased risk of developing deep venous thrombosis (DVT) [18, 19]. Therefore, they should be encouraged to stand and walk during long flights every 1–2 h while awake and perform seated dorsiflexion/plantarflexion exercises to avoid venous stasis that could potentiate clot formation. Staying well hydrated throughout the flight may also decrease the risk of DVT formation.

Those with diabetes are at an increased risk of developing deep venous thrombosis, so they should be encouraged to stand and walk during long flights every 1–2 h and perform seated dorsiflexion/plantarflexion exercises to avoid venous stasis; also, one must remain well hydrated.

Train travel/road travel

In general, traveling by train and/or road is a much more flexible option for a person with diabetes, especially with respect to diet and medications. However, it is mandatory to have a visit with the HCP pretravel and it is most definitely beneficial to know details about the destination beforehand, as listed above.

Since train/road travel is feasible only within the country, specific travel health insurance is not a pre-requisite; but it would be helpful to review one's medical coverage policies and get a list of hospitals/clinics wherein their current insurance will be accepted, at the destination, should unforeseen emergencies arise.

The list of what to pack remains the same, as above.

For carrying and storage of insulin, as mentioned above, FRIO® Insulin Wallet may be a good option [12].

Train travel is much more flexible; though health insurance is not required, one must pack the same essentials in carry-on bags.

Recommendations after arriving at travel destination

- Physical activity

  Depending on travel itineraries, there may be an inadvertent increase in walking more than one is accustomed to (whether at their destinations or in airports between security and boarding gates). This increase in physical exercise may increase glucose utilization and lower blood sugars in addition to more rapid insulin absorption. In such situation, it may be useful to slightly decrease the insulin dosages or eat more carbohydrates and snack between meals to keep blood glucose levels appropriately controlled [20]. It is also advisable to check blood sugar levels more frequently, to be able to keep a track on overall glycemic control. This is considering exposure to a new cuisine, a new environment, and a potentially different physical activity levels [13].

  With increased walking comes the need for comfortable footwear since blisters and abrasions can develop from improperly fitted shoes. Wearing sandals on beaches to reduce the introduction of bacteria and other stray objects is advisable [21].

During travel, there will be an inadvertent increase in walking, for which insulin requirement will decrease; frequent snacking may also help. Blood sugar must be measured more frequently. One must wear proper footwear to avoid ulcers and infections.

- Keeping hydrated

  It is important to remain hydrated, especially when traveling to hotter climates. It is also important to know the quality of the potable water available at one's destination to avoid traveler's diarrhea and the ensuing dehydration [22, 23].

One must be properly hydrated and must also avoid diarrhea.

Conclusion and future perspective

Travelers with diabetes can face challenges during their trips, particularly international travelers. In general, traveling within the country in the same time zone, be it by air or train, poses less of a challenge than traveling outside the country, to a destination with a different time zone.

Being prepared by planning, in advance, will be helpful to achieve management of diabetes and boost self-confidence. This is of utmost importance to achieve appropriate patient glucose control amidst changing diet, time zone difference, and a new environment. Patients should meet their treating physician at least 1 month prior to travel, to allow time for the physician to generate a travel letter and/or prescriptions for needed medications, equipment, and supplies.

Diabetes is manageable when patients and their providers work together to formulate a treatment plan for travel. No destinations should seem "off-limits" to individuals with diabetes, given the available resources that can be utilized in preparation for travel. It is always advisable to take extra precaution while traveling to high altitude (above 8000 ft.) as low oxygen level there may offset glycemic control.

While the above guidelines outlined here seem reasonable, there is no information on how many patients actually have any knowledge of the basics or seek pretravel counseling, and the area remains largely understudied. More data on the diabetic traveling population is needed so that better evidence-based guidelines can be developed.

Tips for safe trips

1. Plan your tour well in advance. Consult your physician and discuss it in details about tour schedule.
2. Carry the prescription, important documents, and a list of all the supplies at hand.
3. Always carry insulin/medicines/accessories double the required amount for you.
4. Use comfortable shoes; always carry some snacks/glucose tabs or gel while on move.
5. Remain hydrated, avoid unaccustomed food and physical activities, and avoid alcohol in excess.
6. Always take help from co-traveler or travel agents in case of emergency.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):11–19
DOI 10.1007/s13410-017-0558-1

REVIEW ARTICLE

Current advances in the utilization of nanotechnology for the diagnosis and treatment of diabetes

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Received: 26 December 2016 / Accepted: 17 April 2017 / Published online: 28 April 2017

Abstract Diabetes is a prevalent disease throughout the world, and the incidence of diabetes is continuously rising. Researchers are constantly searching for advanced technologies for the diagnosis and treatment of diabetes. Nanotechnology is in the forefront of technologies that are being evaluated as an important tool that may serve as reliable methodology in the early diagnosis and treatment of diabetes. Experts in the nanotechnology researcher area are trying to develop novel glucose detection methods and newer delivery methods for insulin and other therapeutics. There has been progress in glucose sensing and insulin delivery; however, this is still at its early development stage. While nanotechnology has been employed in the diagnosis and treatment of a variety of diseases, the present review provides a brief update of the current progress in the utilization of nanotechnology for the diagnosis and treatment of diabetes.

Keywords Diabetes · Nanotechnology · Diagnosis · Therapy

Introduction

Diabetes is a major metabolic disorder that has been estimated to affect more than 366 million people worldwide, and this number is predicted to rise up to more than 552 million by 2030 [1]. According to official reports, Saudi Arabia is among the top 10 countries in the world with highest prevalence of diabetes and may remain in the top 10 countries beyond 2030 [1].

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The application of nanotechnology in the diagnosis and treatment of diabetes will facilitate the use of small volumes of analytes to detect glucose levels and can access the internal cellular areas that are clinically relevant to treat the disease effectively. Additionally, nanotechnology provides miniature materials that are highly durable and with excellent electrical conductivity for glucose sensing [2]. Nanotechnology is also aiding in the closed loop delivery of insulin where insulin is delivered as per the glucose levels automatically. This phenomenon is discussed in detail in the following sections.

This review is focused on the developments in the diagnosis and treatment of diabetes with the aid of nanotechnology. Currently available treatment and advances in the nanotechnology-based therapies were discussed. Strategies like artificial pancreas were also discussed.

Diabetes and its complications

Diabetes mellitus is a chronic disorder characterized by high blood glucose levels that are due to insulin resistance or insulin deficiency. Insulin is a peptide secreted by the β cells, which regulates glucose levels in the blood stream. Diabetes is classified into four categories, namely type 1 diabetes, type 2 diabetes, gestational diabetes, and diabetes due to other causes [3]. Classification of diabetes is important for the determination of therapy. Type I diabetes is also known as insulin-dependent diabetes or juvenile diabetes, which is due to the scarcity of insulin in the body due to the loss of β cell mass in the islets of Langerhans in the pancreas [4].

In type 2 diabetes or the insulin-independent diabetes, the body lacks proportional response to insulin that is secreted by the body, and this type of diabetes is thought to also lead to type 1 diabetes eventually [5]. This type of diabetes has a higher prevalence, when compared to the type 1 diabetes,

which is known as a lifestyle disorder most often accompanied with obesity, inactiveness, or lack of physical activity. Traditionally, it was thought that the type 1 diabetes affects children and type 2 diabetes affects adults, but now, it is ruled as no longer valid by the American Diabetes Association [3].

The third type of diabetes is gestational diabetes, which develops during pregnancy in women and is not overt diabetes, which is diagnosed in the second or third trimester of the pregnancy. If the diabetes is diagnosed in the first trimester, it is called as type 2 diabetes. The fourth category of diabetes or the other specific types of diabetes are understood to be caused by other factors like cystic fibrosis or are induced by chemicals [6]. These include monogenic diabetic syndromes such as neonatal diabetes and maturity onset diabetes of the young (MODY), diseases of the exocrine pancreas such as cystic fibrosis, and drug or chemical-induced diabetes like with glucocorticoid use.

After few years of diabetes, diabetic complications are developed if the blood glucose levels are not rigorously controlled by the patient [7, 8]. Diabetic complications affect most of the organs in the body ranging from the heart, kidneys, and eyes. These complications include acute and chronic complications that need to be treated additionally to the treatment of hyperglycemia. Long-term complications of diabetes include macrovascular atherosclerotic disease including cardiac, cerebral, and peripheral vascular disease and microvascular complications, namely retinopathy, nephropathy, and neuropathy [9].

Cardiovascular disease is similar in diabetic as well as non-diabetic patients, but the frequency is higher in diabetics [9]. Retinopathy occurs in all forms of diabetes, and its development depends on the duration of the disease [10]. Nephropathy unlike retinopathy is developed only in 35–45% of type 1 diabetic patients and less than 20% of the type 2 diabetic patients. However, nephropathy is associated with greatest mortality [11, 12]. Clinical manifestation of neuropathy is variable, and the principal risk posed by the neuropathy is foot trauma and diabetic ulcers [9].

Role of nanotechnology in diagnosis, prevention, and treatment of diseases

Nanotechnology has been shown to have applications in the detection and treatment of various diseases. Nanotechnology-based products have wide range of indications including emesis, infectious diseases, autoimmune diseases, lipid regulation, and immunosuppression (Table 1) [13]. Also, nanoformulations have extensively been evaluated in many research areas including infectious diseases, neurodegenerative diseases, lung diseases, skin diseases, and diabetes as well as many other areas [14–18].

Nanostructured contrast agents were used for target specificity and optimal sensitivity for cancer diagnosis in various imaging techniques like optical imaging (fluorescence and bioluminescence), radionucleotide-based imaging, and computer tomography or magnetic resonance imaging [19]. Nanodiagnostic agents are also utilized in other disease diagnosis like coronary artery disease [20]. Nanodiagnostic agents used in diabetes are discussed in "Utilizing nanotechnology for the diagnosis of diabetes" section.

In drug delivery, nanotechnology aids in drugs that have suffered poor bioavailability problems or that resulted in adverse effects when delivered through conventional dosage forms. Amphoterecin B is the best example for the improvement of bioavailability and efficacy by the nanodrug delivery system liposome [21]. Another drug doxorubicin has no bioavailability problem but resulted in serious cardiotoxicity; however, the toxicity has been circumvented by the liposomal formulation [22]. Nano-sized aprepitant has no effect of food on the dissolution rate which, in turn, affects the bioavailability [23]. Drugs of biological origin like enzymes and aptamers like adenosine deaminase and interferon 2a are conjugated with polymers to enhance the efficacy of these drugs [13]. These polymer conjugates fall in the nano-sized range and are considered nanodrug delivery systems. Vaccines like hepatitis A were developed as virosomes, a form of nanodrug delivery system to enhance the uptake of the vaccine by immune cells [24]. In some cases, nanodrug delivery systems are utilized to make use of the abnormal physiology of the affected site for the targeting of drugs like in cancer and some ocular diseases. Poly(styrene-co-maleic) acid conjugated to neocarzinostatin (SMANCS) is a drug that utilizes the said phenomenon in cancer that is known as enhanced permeation and retention (EPR) effect [25].

There are various types of nanotechnology-based drug delivery systems including polymer drug conjugates, micellar formulations, liposomes, nano-sized drug particles, and protein-bound drugs (Fig. 1). Apart from these nanoparticles, self-emulsifying drug delivery systems and polymersomes are under late research stages [13]. Development of drug delivery systems for these diseases relies on the pathology of the disease. In cancer, nanodrug delivery systems help in passive targeting of the tumor by the enhanced permeation and retention effect [25]. Sometimes, the drug delivery systems help in avoiding the adverse reactions caused by the conventional therapy as in the case of doxorubicin [26].

Utilizing nanotechnology for the diagnosis of diabetes

Early detection of diabetes and estimating the disease progression are the important aspects of diabetes management. Diabetic patients need to monitor their blood glucose levels constantly to regulate the levels and to have tight control to delay the onset of diabetic complications [7, 8]. The timeline

Table 1 Nanotechnology-based drug delivery systems in various diseases

Indication	Products	Drugs	Delivery system
Fungal infections	Abelcet	Amphoterecin B	Lipid complex
	Amphotec	Amphoterecin B	Lipid colloidal dispersion
	Ambisome	Amphoterecin B	Liposomes
Cancer	Doxil/Caelyx	Doxorubicin	Liposomes
	Daunoxome	Daunorubicin	Liposomes
	Depocyt	Cytarabine	Liposomes
	Abraxane	Paclitaxel	Paclitaxel albumin-bound particles
Multiple sclerosis	Copaxone	Copolymer of alanine, lysine, glutamic acid, and tyrosine	Polymer
Hepatitis A	Epaxal Berna	Hepatitis vaccine	Virosome
Rheumatoid arthritis	Cimzia	PEGylated antibody (anti-TNF Fab′)	Polymer conjugate
Immunodeficiency	Adagen	PEG-adenosine deaminase	Polymer conjugate
Antiemetic	Emend	Aprepitant	Nano-sized drug
Menopausal therapy	Estrasorb	Estradiol	micellar nanoparticles
Atypical antipsychotic	Invega Sustenna	Paliperidone palmitate	Nano-sized drug
Breast cancer	Myocet	Doxorubicin	Liposomes
Febrile neutropenia	Neulasta	PEG-G-CSF	Polymer conjugate
Leukemia	Oncaspar	PEG-asparaginase	Polymer conjugate
Hepatitis C	Pegasys	PEG-interferon 2a	Polymer conjugate
	PEG-Intron	PEG-interferon 2b	Polymer conjugate
Age-related macular degeneration	Macugen	Pegylated anti-VEGF aptamer	Polymer conjugate
Anemia	Mircera	PEGylated epoetin beta	Polymer conjugate
Acromegaly	Somavert	PEG-HGH	Polymer conjugate
Chronic kidney disease	Renagel	Cross-linked poly(allylamine) resin	Polymer conjugate
Eating disorders	MegaceES	Megesterol acetate	Nanocrystals
Immunosuppressant	Rapamune	Sirolimus	Nanocrystals
Lipid regulation	Tricor Lyphantyl	Fenofibrate	Nanocrystals
Age-related macular degeneration	Visudyne	Verteporfin	Liposomes

Modified from reference [13]

of glucose monitoring is given in Fig. 2. The popular diagnostic tools utilize the blood drop collected after pricking the finger with needle. However, this method is painful to read the blood glucose levels often, so to avoid these issues, glucose monitoring devices have been developed that use canulae or sensors that are placed beneath the skin to monitor blood glucose multiple times with a single insertion [27]. Most of the developments have been in the direction of continuous real-time monitoring of the blood glucose levels utilizing advanced technology. Real-time measurement of glucose is highly

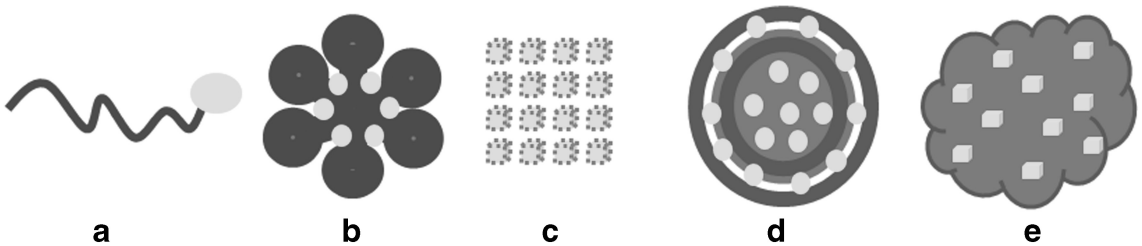

Fig. 1 Various types of nanodrug delivery systems that have been marketed. **a** Polymer drug conjugates. **b** Micellar formulations. **c** Nano-sized drug particles. **d** Liposomes. **e** Protein-bound drugs

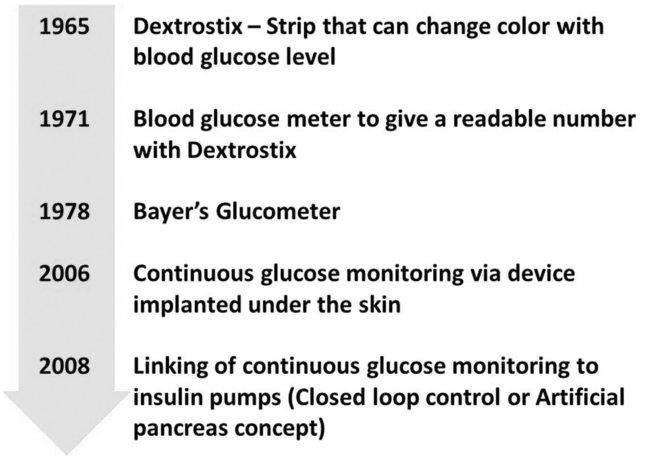

Fig. 2 Timeline of glucose monitoring

beneficial for diabetic patients. Glucose sensors are used to monitor glucose levels in the blood or interstitial fluid [28]. A glucose sensor will have three components namely a detector, a transducer, and a reporter. The need for betterment of glucose sensors is to improve the accuracy and specificity and to achieve real-time detection [29].

Nanotechnology has a notable impact on the glucose sensors as this technology provides increased surface area for the sensors and increased catalytic activity of the electrodes and in envisioning miniature nanoscale devices for the detection of glucose [30]. The limitations of surface area, catalytic activity, and size of the devices were all overcome by the advent of nanotechnology. Surface-enhanced Raman scattering-based glucose biosensor using silver film over nanospheres (AgFON) is under research stage and is intended to be a nanoscale implant under the skin [31]. This is thought to provide minimally invasive real-time glucose sensing that is highly advantageous in diabetic patients. Nanoparticle-based glucose sensors rely on three types of molecules, namely enzymes used in glucose detection like glucose oxidase, glucose binding proteins, and glucose binding small molecules [32]. The nanoparticles when used as transducers provide patient friendly, precise, and rapid glucose sensors for real-time measurements by helping to achieve large signal enhancements and lower limits of detection [33], The first generation of glucose sensors utilize amperometric glucose oxidase sensing technology [34]. Glucose oxidase is highly specific for glucose which reacts with blood glucose or urinary glucose to convert it to D-glucono-δ-lactone and hydrogen peroxide [35]. The oxidation of glucose results in electric current that is proportional to the glucose concentration [36].

Glucose oxidase has been inserted into nanoparticles of various types including palladium, gold, platinum, and carbon nanotubes [30]. The first generation of glucose sensors depend on the oxygen as co-substrate, and detection of glucose is interfered by the presence of electroactive species in the blood. To overcome these drawbacks, second-generation glucose sensors were developed that utilize synthetic electron acceptors as alternative to co-substrates. These also posed some problems with respect to the size of the co-substrates [37].

The third-generation sensors were developed without employing any electron mediators and were able to transfer the electron from the enzyme directly to the electrode [38]. However, there remain certain drawbacks of using enzymes like batch to batch variability and diminished activity of enzyme with time [30]. Thus, non-enzymatic glucose sensors have been developed to avoid these limitations [39]. One type of non-enzymatic glucose sensor utilizes metal catalysts like copper oxide or gold nanoparticles to oxidize glucose [40].

Another type of non-enzymatic glucose sensor relies on binding of glucose to give voltammetric [33] or fluorescent readout [41]. In these sensors, glucose displaces water from the binding pocket which results in shift in electron density that can be measured as voltammetric or fluorescent output. These types of sensors do not require battery and can serve continuously [42]. Nanoparticles such as quantum dots and single-walled carbon nanotubes were used as fluorescence emitting components of these sensors [43]. Due to their miniature size, these sensors can be used at multiple sites and can provide accurate readings when compared to a single sensor. The detection limit of glucose levels has also been improved by the use of nanotechnology that is evident from a study using amperometric oxidation of glucose on nickel oxide nanoparticles that are deposited on DNA-coated glassy carbon electrode which showed a detection limit of 17 nM [44].

Apart from glucose level detection, detection of β cell mass is also very important in type 1 diabetes. As the diabetes progresses, there is a loss of β cell mass which is a characteristic of the disease. Estimating the extent of loss of β cell mass will provide an understanding of how much insulin needs to be administered. However, direct estimation of β cell mass is impractical and can only be done on the excised tissue [32]. Fortunately, nanotechnology can now be applied to measure β cell mass by using nanoprobes that have high specificity for them, which will enable clinicians to quantify the β cell mass non-invasively [45]. Super paramagnetic iron oxide nanoparticles that target β cells were developed by attaching the targeting moieties like modified exendin-4 peptide to detect the β cell mass non-invasively using magnetic resonance [46].

Furthermore, nanofibers have also been used in the detection of glucose levels. These nanofibers have been manufactured mostly utilizing electrospinning technology [37] and layer by layer technique, which involves the electrostatic deposition of alternating layers of positively and negatively charged polymers [47]. The latter approach develops very thin film with tunable permeability and controlled biocompatibility [48]. This technique will lead to a high activity surface with minimum possible thickness and also protects the loaded material from external factors and enzymes like proteases. Generally, a thickness of 10 nm is achieved for six

bilayers, which is then implanted into the subcutaneous tissue as smart tattoos [49]. This film allows the passage of glucose inwards and protects the sensor material from damage. If the sensing mechanism involves near-infrared-based fluorescence assay, glucose sensors can be excited and the fluorescence reading can be read outside the body making it non-invasive [50].

Treatment of diabetes

Conventional treatment of diabetes

Diabetes has no cure, but it can be managed by available therapies. The treatment relies on insulin and other antidiabetic agents' delivery. The conventional form of insulin replacement therapy is open loop that relies on the historical understanding of the patient's unique blood glucose profile [50]. Microcomputer-based devices which are under research stage include glucose monitoring, and insulin delivery combined can deliver the required amount of insulin after calculating the required dose automatically in a closed loop manner. Most of the antidiabetic drugs are delivered orally, but insulin is delivered invasively. There is a lag period in the conventional therapy for diabetes which accounts for the time period after detection of the glucose levels and administration of insulin and its delayed absorption through subcutaneous route. This lag period sometimes accounts for the hypoglycemia observed in diabetic patients. Nanotechnology-based treatment is supposed to detect the glucose levels in real time and deliver insulin as and when the body requires it thus avoiding lag time and occasions of hypoglycemia. Due to the advancements in detection of glucose in real time aided by subcutaneous implants that are used in closed loop devices as discussed in "Utilizing nanotechnology for the diagnosis of diabetes" section and delivery of insulin as and when required the lag time is minimized and the deleterious effects of hyperglycemia are surpassed.

Diabetes is treated based on the etiology of the disease. For type 1 diabetes, insulin replacement therapy is prescribed. Insulin can be given as multiple dose injections or can be given as continuous subcutaneous infusion using an insulin pump. Other than insulin, type 1 diabetes can be treated with insulin analogs and pramlintide. Pramlintide is an amylin analog that blunts the glucagon secretion and enhances satiety. This treatment is indicated only in adults and has been shown to produce weight loss and reduction in the requirement of insulin dose. For type 2 diabetes, metformin treatment is preferred; if there were no benefits observed in terms of glycated hemoglobin levels, the treatment is shifted to dual therapy with other antidiabetic drugs, and similarly, if the dual therapy does not affect the glycated hemoglobin, then a triple therapy is preferred, even if this is not effective; then, combination injectable therapy is prescribed [51]. Along with metformin drugs belonging to classes like sulfonyl ureas, thiazolidine diones, dipeptidyl peptidase 4 inhibitors, sodium/glucose cotransporter 2 inhibitors, or glucagon like peptide 1 agonists are used in the treatment of type 2 diabetes currently (Table 2).

Insulin delivery using nanotechnology

For the treatment of type 1 diabetes and advanced type 2 diabetes, insulin replacement therapy is prescribed. Insulin formulations are available in long-acting and short-acting forms and are given subcutaneously using a needle. Administration of insulin by multiple injections results in poor patient compliance to these formulations [52]. Insulin is also being delivered through infusion pumps for better patient compliance but is available only in few developed countries. Insulin pumps, nowadays, come with a sensor that will shut off the delivery of insulin for a specific amount of time when the user does not respond to low glucose levels, increasing the safety of these devices.

Since the discovery of insulin, there have been many attempts to deliver insulin in a non-invasive manner. Insulin is delivered invasively, which is the case with most protein drugs [53]. Being protein drug with 51 amino acids, insulin cannot be delivered through oral route due to the harsh conditions encountered in the gut [54]. Moreover, the gut wall acts as a barrier for insulin due to its larger size posing as additional barrier for the delivery of insulin. Taken together, oral delivery of insulin results in its poor bioavailability [55].

There have been efforts like use of permeation enhancers, protease inhibitors, and protein ligand conjugates for the delivery of insulin orally. All these efforts could not result in a beneficial oral formulation till date [54]. The bioavailability of orally delivered drugs is dependent on solubility, permeability, and stability [13]. The size and the nature of insulin do not permit its delivery through oral route. Therefore, to get the advantage through oral route formulation, approach should focus on permeability and stability of the insulin which is the case with all the protein drugs [56].

Currently, there is a significant interest in developing non-invasive methods for the delivery of insulin and/or to prolong the duration of its effect using nanotechnology. Nanomedicine-based insulin delivery involves polymer therapeutics, micelles, liposomes, solid lipid nanoparticles, and nanoparticles of biodegradable polymers [57]. Polymer therapeutics for protein drugs most often include polyethylene glycol (PEG), where peptide or protein drug-like insulin is attached to PEG for increased solubility, permeability, and stability when delivered orally [58]. Similarly, micellar formulations have also shown improvements in the use of insulin through oral route.

However, liposomes are believed to be more suitable and stable forms than micelles, and some forms of liposomes have

Table 2 Current treatment strategies for type 1 and type 2 diabetes [42]

	Type 1	Type 2
Pharmacotherapy	Multiple dose insulin injections	Metformin monotherapy
	Continuous subcutaneous insulin infusion	Dual therapy
	Insulin analogues	Triple therapy
	Pramlintide	Combination injectable therapy
Surgical treatment	Pancreas transplant	Bariatric surgery

The information in the table is gathered from American diabetes association report [42]. In the treatment of type 2 diabetes based on the glycated hemoglobin levels, the dual (metformin + other class of antidiabetic drug) and triple therapy (metformin + two drugs of different classes) are prescribed using different classes of antidiabetic drugs

thus far been developed and tested in animals for the delivery of insulin [59, 60]. Liposomal insulin can be delivered through oral route and was shown to be more bioavailable than free form through this route [60]. These liposomes have shown to protect the insulin from degradation in the gastrointestinal tract [61]. In a study, plant-derived sterols have been used as alternatives to the cholesterol that is widely used in the preparation of liposomes and to encapsulate insulin. The β-sitosterol, stigmasterol, ergosterol, and lanosterol have been used to stabilize the liposomal membrane, of which ergosterol-stabilized liposomes have shown greater capacity to protect the insulin against degradation in the simulated gastric fluids [60]. Furthermore, ergosterol-stabilized liposomes showed a greater stability when compared to the cholesterol-stabilized liposomes, and these were efficient in producing a hypoglycemic effect in normal rats [60]. Liposomes have also been studied for the delivery of insulin through pulmonary route [62].

Nanoparticles are another form of nanodrug delivery system that are being explored for the delivery of insulin. Insulin-loaded nanoparticles have been developed using various polymers like chitosan, polylactide-co-glycolic acid, and dextran [63]. Solid lipid nanoparticles have also been developed for the delivery of insulin [64]. However, effective nanoparticle should be biocompatible, biodegradable, and non-immunogenic and should not be discovered by the mononuclear phagocyte system in the blood stream. Nanoparticles are thought to be absorbed by the lymphatic system in the intestinal wall especially from Peyer's patches [65]. Polymers like chitosan act as permeation enhancers which is beneficial in the absorption of these nanoparticles through paracellular pathway [66]. Chitosan is known to break the tight junctions between the enterocytes to reach the blood stream [67].

There are other types of nanoparticles, mucus-penetrating nanoparticles, which are known to be taken up by the lymphatic system in the small intestine that has a mucus layer over the enterocytes. In order to reach the blood stream, these nanoparticles are designed specifically to cross this thick mucus layer first [68]. Mucoadhesive nanoparticles can sometimes get trapped in this mucus layer and cannot enter the blood stream. Nanoparticles need to cross this layer, and the rate at which they cross the layer should be faster than the mucus turnover rate; otherwise, they will end up in waste [69]. This is true especially for the insulin-like large molecules because these molecules when entrapped in nanoparticles need to be taken up into the blood stream as intact nanoparticles; if the nanoparticles release the insulin in the intestine. it will be degraded by the proteases in the small intestine [70].

Nanotechnology can be used to deliver insulin for prolonged durations [Fig. 3] [71]. Nanodrug delivery systems can also be used as glucose-responsive particles, where high glucose level will trigger the release of insulin [72]. The slow release of insulin offered by the nanoparticles will extend the action of insulin and hence replacing the need for multiple injections that is believed to be a troublesome for patients and thus increasing patient compliance. Insulin nanoparticles can be made as glucose responsive for strict regulation of blood glucose levels; for example, polymers containing boronic acids show solubility dependence on the concentration of diol in the solution; thus, in the presence of sufficient

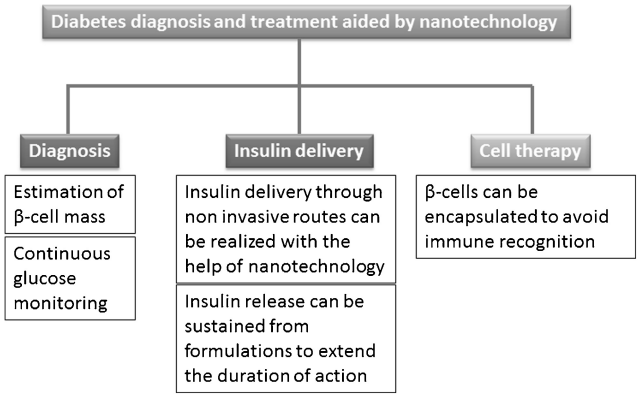

Fig. 3 Potential uses of nanotechnology for the management of diabetes. Nanotechnology has applications in the diagnosis of diabetes in blood glucose monitoring and estimation of β cell mass. In therapy, nanotechnology is being used for the delivery of insulin and other drugs via oral, pulmonary, and other non-invasive routes and for sustained release of insulin/drugs for prolonged durations. Nanotechnology facilitates the application of other strategies like insulin delivery and cell therapy

concentration of the diol, neutral boronic acids will result in the formation of ionized cyclic esters with 1,2 or 1,3 diols, which make them hydrophilic [73]. Therefore, applying the same theory using these polymers as saccharide (especially glucose) detectors, if insulin-loaded nanoparticles or micelles are made up, they will respond to the glucose concentrations to release insulin [74].

Other mechanisms through which the nanoparticles can be made glucose responsive include the combination of pH-sensitive polymers with glucose-sensitive enzyme such as glucose oxidase. These triggers can be integrated within the nanoparticles that disassemble by either swelling or degrading in response to increased glucose levels [32].

Artificial pancreas

Artificial pancreas is a concept of delivering insulin only when there is a requirement by the body through a device that can be implanted in the body [75]. Closed loop delivery of insulin with automated dose calculations is generally considered as an artificial pancreas. It is intended to measure the glucose levels constantly, and then, when the glucose levels are high enough, the insulin is released or pumped into the blood stream. This could be a permanent solution for type 1 diabetic patients. There needs to be a sensor electrode that repeatedly measures the blood glucose, and the information has to be transferred to a small computer that activates the infusion pump to release the required amount of insulin into the blood stream. Artificial pancreas concept relies mostly on continuous glucose monitoring systems and algorithms for the actuation of insulin pump to release insulin [76].

Another concept of artificial pancreas involves the insertion of pancreatic beta cells taken from animals into a small box that is surrounded with small pores that can allow the passage of glucose and insulin [Fig. 3] [77]. These small pores are very useful in preventing the immune system from attacking the beta cells of foreign origin. This can avoid the use of immunosuppressants that will weaken the patients' immune system leaving them with serious risk of infection after pancreas transplantation. Also, the utilization of layer by layer nanofilms will protect the inserted pancreatic cells from immune rejection. Islet cells can be encapsulated into macrocoverings or microcoverings with the intention of avoiding immune cells and proteins while allowing glucose and nutrient access. However, this approach has not seen any success through many years but will remain a possible approach [50].

Conclusion

Nanotechnology is highly beneficial in diabetes as it can help in diagnosis and treatment of diabetes. It is being helpful in the development of new strategy for the treatment of diabetes that includes glucose-responsive insulin therapy. Continuous glucose monitoring devices and insulin delivery concepts like artificial pancreas when realized will be invaluable for diabetic patients. The absence of lag time between glucose detection and insulin delivery will save many patients from hypoglycemia which can be realized with the help of nanotechnology. Non-invasive routes of insulin delivery through various nanodrug delivery systems are actively researched and can be a reality in near future. Nanotechnology is also helping in the development of treatment strategies for diabetic complications. Together, nanotechnology can provide a higher level of patient care in diabetic patients.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):20–25
DOI 10.1007/s13410-016-0545-y

ORIGINAL ARTICLE

Early diagnosis of gestational diabetes mellitus during the first trimester of pregnancy based on the one-step approach of the International Association of Diabetes and Pregnancy Study Groups

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Received: 17 September 2016 / Accepted: 21 December 2016 / Published online: 27 December 2016

Abstract To examine the utility of the 75 g oral glucose tolerance test (OGTT), conducted according to the criteria of the International Association of Diabetes and Pregnancy Study Groups (IADPSG), for the early diagnosis of gestational diabetes mellitus (GDM) and to propose new cut-off values. A total of 350 prospectively enrolled patients were admitted to Inonu University School of Medicine Obstetrics and Gynecology Outpatient Clinic between April 2012 and January 2015 for first-trimester screening. Gestational diabetes mellitus (GDM) during the first trimester of pregnancy (11–13 weeks) was diagnosed using the 75-g OGTT. In patients who tested negative, the OGTT was repeated at 24–28 weeks. GDM was diagnosed in 14.6% of the patients, of whom 80.3% were diagnosed during the first trimester. In these patients, there were no remarkable changes in fasting plasma glucose level when a fasting glucose cutoff of 92 mg/dl was used for the diagnosis of GDM. The sensitivity and specificity of the OGTT were 66.6% and 99.3%, respectively (area under the receiver operating characteristic curve [AUROC] 0.892, 95% CI 0.855–0.923, $p < 0.001$). The cutoff value for a positive 75-g OGTT result was reduced from 180 to 173 mg/dl for the 1-h post-glucose load (AUROC 0.908, 95% CI 0.873–0.936, $p < 0.001$) and from 153 to 129 mg/dl for the 2-h post-glucose load (AUROC 0.861, 95% CI 0.515–0.775, $p < 0.001$). The 75-g OGTT based on IADPSG criteria can be used to detect 80% of GDM cases as early as the first trimester. A modification of current cutoff values would improve the sensitivity of the test but lower its specificity.

Keywords Early diagnosis · Gestational diabetes · Hyperglycemia · Oral glucose tolerance test · Pregnancy

Introduction

Gestational diabetes mellitus (GDM) is defined as a carbohydrate intolerance detected for the first time during pregnancy. Although the screening and diagnosis of GDM remain controversial, GDM is one of the most common complications of pregnancy [1]. It is also associated with an increased risk of maternal and perinatal complications, such as preterm delivery, gestational hypertension, preeclampsia, macrosomia, increased risk of cesarean delivery, shoulder dystocia, neonatal hyperbilirubinemia, and respiratory distress syndrome [2]. In 2010, based on the results of the Hyperglycemia and Adverse Pregnancy Outcome study, which specifically evaluated the relationship between maternal glucose levels and maternal, perinatal, and neonatal outcomes, the International Association of Diabetes and Pregnancy Study Group (IADPSG) recommended new criteria for the diagnosis of GDM based on a universal 75-g, 2-h oral glucose tolerance test (OGTT) performed during pregnancy. Patients in whom any single fasting, 1 h, or 2 h value exceeded a certain threshold value are considered positive for GDM [3].

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The screening and diagnosis of GDM are traditionally performed at 24–28 weeks gestation, given the increased diabetogenic effect of pregnancy during the late second trimester [4]. Early GDM screening during pregnancy is recommended only for women with undiagnosed type 2 diabetes or with risk factors, including a prior history of GDM, known glucose metabolic impairment, and obesity [5]. Other authors have advocated an alternative GDM screening strategy for earlier testing, adjusting the established criteria of the test accordingly [6]. An effective definition of patients at high risk of GDM would allow early dietary advice and pharmacological interventions, thereby improving pregnancy outcomes and reducing GDM-related maternal and perinatal complications.

The aim of this study was to examine the utility of the 75-g OGTT for the early diagnosis of GDM. Based on the results, we propose new cutoff values for this test to allow an early diagnosis of GDM during the first trimester of pregnancy.

Materials and methods

The study was approved by the Ethics Committee of Inonu University Faculty of Medicine (no: 2012/4-1). Verbal and written information was provided to all study participants in accordance with the principles of the Declaration of Helsinki, and informed consent was obtained from all patients prior to their enrollment. The study population consisted of 350 prospectively enrolled patients who were admitted to Inonu University School of Medicine Obstetrics and Gynecology Outpatient Clinic between April 2012 and January 2015 for first-trimester GDM screening. GDM was diagnosed during the first trimester of pregnancy (11–13 weeks) based on a positive 75-g OGTT result, as defined by the IADPSG one-step diagnostic approach. After fasting for 8–10 h, the patients were requested to drink a solution containing 75 g glucose, followed by measurement of the venous plasma glucose concentration 1 and 2 h after ingestion. The diagnosis of GDM was defined based on a single glucose concentration that met or exceeded the threshold value (fasting value, 92 mg/dl; 1-h value, 180 mg/dl; and 2-h value, 153 mg/dl). Patients with a negative test result during the first trimester were tested again during the second trimester (24–28 weeks). Patients with pregestational diabetes, multiple pregnancies, pregnancies with major fetal anomalies (fatal or requiring prenatal and postnatal surgery), fetal death, chromosomal abnormalities, or genetic syndromes were excluded from the study.

The data are presented as means and standard deviations or as medians and interquartile ranges. The Shapiro-Wilk test was used to analyze data with a normal distribution, and one-way analysis of variance was used to compare the three groups (GDM-negative group, GDM diagnosed during the first trimester, and GDM diagnosed during the second trimester). The Bonferroni test was used for multiple comparisons among the groups. The GDM-negative and late-onset (second trimester) GDM groups were compared using the Mann-Whitney *U* test. Area under the receiver operating characteristic curve (AUROC) analysis was performed to examine the performance of the accepted cutoff values proposed by the IADPSG during the first trimester and to determine new cutoff values predictive of first-trimester GDM. The optimum cutoff values were identified using the Youden index. A p value <0.05 was considered to indicate statistical significance. SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) was used for all data analyses.

Results

First-trimester GDM screening was performed in 350 patients. Of the 51 patients diagnosed with GDM, 41 (80.3%) had a positive OGTT result during the first trimester and 10 (19.7%) during the second trimester. Patient testing at 11–13 weeks resulted in an 11.7% positivity rate according to the IADPSG criteria. In the GDM-negative patients, the OGTT was repeated at 24–28 weeks, and 2.8% of these retested patients were positive for GDM. Thus, of the 350 patients who were screened, 14.6% had GDM, 80.3% of whom were diagnosed during the first trimester (Fig. 1).

The median age of the patients with early-onset (i.e., first trimester) GDM was 32.80 ± 6.98 years, which was significantly older than that of patients in the GDM-negative group ($p = 0.011$). Patients in the early-onset GDM group also had a significantly higher body mass index (26.90 ± 5.68) than that of GDM-negative patients ($p = 0.002$). Compared with patients in the early-onset GDM and GDM-negative groups, patients in the late-onset (second trimester) GDM group were not significantly different in terms of age or BMI. Table 1 summarizes the maternal and pregnancy characteristics of the three groups.

The fasting plasma glucose (FPG) level and the plasma glucose levels at 1 and 2 h after administration of the 75-g glucose load at 11–13 and 24–28 weeks gestation are shown in Table 2 for the 299 patients without GDM, the 41 patients with early-onset GDM, and the 10 patients with late-onset GDM. At 11–13 weeks, the median FPG level and the 1- and

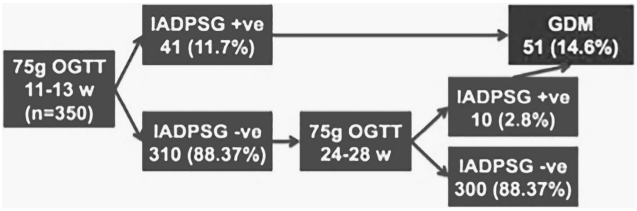

Fig. 1 A flow chart summarizing the diagnosis of GDM at 11–13 and 24–28 weeks gestation

Table 1 Maternal and pregnancy characteristics of groups

Maternal characteristics	Normal (n = 299)	Early-onset GDM (n = 41)	Late-onset GDM (n = 10)
Age	30.12 ± 5.39[a]	32.80 ± 6.98[b]	32.20 ± 5.53[ab]
Gravidy	2.42 ± 1.54[a]	3.24 ± 1.92[b]	3.50 ± 2.12[ab]
Parity	1.00 ± 1.06[a]	1.48 ± 1.59[b]	1.30 ± 1.05[ab]
Abortus	0.41 ± 0.92[a]	0.65 ± 0.96[ab]	1.20 ± 1.54[b]
BMI (kg/m^2)	24.25 ± 4.12[a]	26.90 ± 5.68[b]	24.50 ± 5.64[ab]
History of GDM	4 (1.3)[a]	3 (7.3)[a]	0 (0)[a]
DM in first degree relatives	1 (0.3)[a]	0 (0)[a]	0 (0)[a]
History of having macrosomic fetus	10 (3.3)[a]	3 (7.3)[a]	0(0)[a]
Mode of previous delivery			
Nulliparous	124 (41.5)[a]	11 (26.8)[a]	1(10)[a]
Vaginal	85 (28.4)[a]	15 (36.6)[a]	5(50)[a]
Cesarean	90 (30.1)[a]	15 (36.6)[a]	4 (40)[a]

Each subscript letter denotes a subset of group categories whose row proportions do not differ significantly from each other at the 0.05 level

2-h post-glucose load levels were significantly higher in the early-onset GDM group than in the GDM-negative and late-onset GDM groups. Also, during the first trimester, the median FPG and 2-h post-glucose load levels were similar between the GDM-negative and late-onset GDM groups, whereas the 1-h post-glucose load level was significantly higher in the late-onset GDM group than in the GDM-negative group.

Among the 51 patients diagnosed with GDM either in the first or second trimester, AUROC analysis was used to examine the performance of the IADPSG cutoff values in predicting first-trimester GDM and to determine new cutoff values predicting first-trimester GDM. The positivity rate, sensitivity, and specificity of the 75-g OGTT during the first trimester, according to the IADPSG cutoff values and the new cutoff values determined by AUROC analysis, are shown in Tables 3 and 4, respectively. There were no remarkable changes in terms of fasting plasma glucose level in the FPG cutoff value of 92 mg/dl [sensitivity 66.6%, specificity 99.3%; AUROC 0.892, 95% confidence interval (CI) 0.855–0.923, $p < 0.001$] for the diagnosis of GDM during the first trimester. The cutoff value for a positive 75-g OGTT result was reduced from 180 to 173 mg/dl for the 1-h post-glucose load (AUROC 0.908, 95% CI 0.873–0.936, $p < 0.001$) and from 153 to 129 mg/dl for the 2-h post-glucose load (AUROC 0.861, 95% CI 0.515–0.775, $p < 0.001$). Lowering the thresholds to 173 and 129 mg/dl for the 1- and 2-h post-glucose loads, respectively, improved the sensitivity from 60.7 to 70.4% and from 52.9 to 76.4%, respectively, but reduced the specificity of the test from 100 to 97.3% and from 99 to 91.3%, respectively. The AUROC values predicting GDM using the 75-g OGTT at 11–13 weeks gestation are shown in Fig. 2.

During the antenatal period, preeclampsia and preterm birth rates were similar among the three groups, as were gestational age at delivery and the frequency of cesarean delivery. Neonatal birth weight was significantly higher in the early-onset GDM group than in the other two groups. The macrosomia rate and 1- and 5-min Apgar scores were similar among the three groups, as were the rates of neonatal

Table 2 Results of the 75-g OGTT, which included the measurement of the plasma glucose level before and after 1 and 2 h oral administration of 75 g of glucose, at 11–13 and 24–28 weeks gestation (median and IQR in parentheses)

	Normal (n = 299)	Early-onset GDM (n = 41)	Late-onset GDM (n = 10)	p value
Fasting glucose levels (mg/dl)				
11–13 weeks	79 (76–84)[a]	99 (95–130)[b]	81 (78–85)[a]	<0.001
24–28 weeks	81 (77–87)[a]	–	93 (93–95)[b]	<0.001
1-h post-glucose level (mg/dl)				
11–13 weeks	134 (106–151)[a]	188 (180–202)[b]	152(135–177)[c]	<0.001
24–28 weeks	144 (131–156)[a]	–	186.5 (181–191)[b]	<0.001
2-h post-glucose level (mg/dl)				
11–13 weeks	101 (88–111)[a]	160 (148–166)[b]	105 (93–134)[a]	<0.001
24–28 weeks	106 (97–121)[a]	–	165.5 (151–166)[b]	<0.001

Each subscript letter denotes a subset of group categories whose row proportions do not differ significantly from each other at the 0.05 level

Table 3 The positive rate, sensitivity, and specificity of the IADPSG cutoff values of the 75-g OGTT in the first trimester GDM prediction after ROC curves analysis

	Cutoff point	Positive rate (%)	Sensitivity (95% CI)	Specificity (95% CI)
Fasting glucose (mg/dl)	92	9.4	66.6 (52.1–79.2)	99.3 (97.6–99.9)
1-h post-glucose level (mg/dl)	180	8.2	60.7 (46.1–74.2)	100 (98.8–100.0)
2-h post-glucose level (mg/dl)	153	7.4	52.9 (38.5–67.1)	99.0 (97.1–99.8)

hypoglycemia and requirement for the neonatal intensive care unit. There were no cases of perinatal mortality. The perinatal and neonatal outcomes of the patients are shown in Table 5.

Discussion

In this study, the 75-g OGTT was successful in detecting 80% of first-trimester GDM cases according to the IADPSG criteria. Our results showed that after oral administration of 75 g glucose, the cutoffs for 1- and 2-h blood glucose levels during first-trimester testing should be 4–15% lower than those during late second-trimester testing. Maegawa et al. [7] performed four screening tests in patients during their first trimester of pregnancy: a 50-g oral glucose challenge test, a casual plasma glucose measurement, measurement of glycosylated hemoglobin levels, and an FPG assay. After 2–4 weeks, they performed a 75-g OGTT as a diagnostic test in the same patients. Of the 749 pregnant women enrolled in the study, 22 (2.9%) were diagnosed with GDM. Consistent with the results of our study, the majority (14/22, 63.6%) of the GDM cases were detected during the first trimester. Dashora et al. [8] evaluated 564 patients during the first trimester of pregnancy for glucose intolerance using the 75-g OGTT. Patients with normal results were retested two or three times over 2-month intervals, with the last test conducted during the seventh month of gestation. Abnormal glucose tolerance was detected in 21.3% of pregnant women, 88% in whom GDM was detected before the seventh gestational month. In that study, 10% of the women required a second test, and 2.5% were not diagnosed until after the third test. The authors suggested that a significant percentage of cases can be detected early in pregnancy rather than waiting until 7 months gestation, as has been performed traditionally. They also concluded that early and multiple screenings for GDM can improve the detection of GDM and positively influence pregnancy outcomes. Our study further demonstrated that our three groups of patients did not differ significantly in terms of perinatal and neonatal outcomes. This is attributed, at least in part, to the implementation of preventative measures in the early-onset GDM group, enabling a reduction in the adverse effects of hyperglycemia during an earlier stage of pregnancy.

Several reports have suggested the utility of FPG measurements in GDM screening. Reichelt et al. [9] showed that in diabetes screening, a FPG level of 89 mg/dl maximizes both the sensitivity (88%) and specificity (78%) of the tolerance test, as 22% of the women in their study tested positive according to this criterion. Agarwal et al. [10] suggested that the variation in performance of FPG measurements observed among many studies may be due to the various diagnostic criteria used. They compared the effect of four different diagnostic criteria applied to the same 75-g OGTT values in a cohort of 4602 pregnant women and concluded that FPG was very useful in GDM screening based on the American Diabetes Association guidelines as the diagnostic criteria (as applied to the 75-g OGTT). The FPG measurements obtained in the present study afforded a sensitivity and specificity of 66.6 and 99.2%, respectively, during the first and second trimesters, using a FPG cutoff value of 92 mg/dl. In addition, AUROC analysis did not lead to remarkable changes in the FPG cutoff value used for the diagnosis of first-trimester GDM.

The increase in the plasma glucose concentration from before to after the 75-g OGTT was greater at 24–28 weeks than at 11–13 weeks, in both the GDM-negative and late-onset GDM groups. This finding confirms the well-described diabetogenic effect of pregnancy, which increases with gestation

Table 4 The positive rate, sensitivity, and specificity of the proposed cutoff values of the 75-g OGTT in the first trimester GDM prediction after ROC curves analysis

	AUC (95% CI)	Criterion (95% CI)	Positive rate	Sensitivity (95% CI)	Specificity (%95 CI)	*p*
Fasting glucose (mg/dl)	0.892 (0.855–0.923)	>92 (>81– > 94)	9.4	66.6 (52.1–79.2)	99.3 (97.6–99.9)	<0.0001
1-h post-glucose level (mg/dl)	0.908 (0.873–0.936)	>173 (>157– > 177)	12.2	76.4 (62.5–87.2)	97.3 (94.8–98.8)	<0.0001
2-h post-glucose level (mg/dl)	0.861 (0.820–0.895)	>129 (>123– > 149)	16.8	76.4 (62.5–87.2)	91.3 (87.5–94.2)	<0.0001

AUC area under curve, *CI* confidence interval, *h* hour

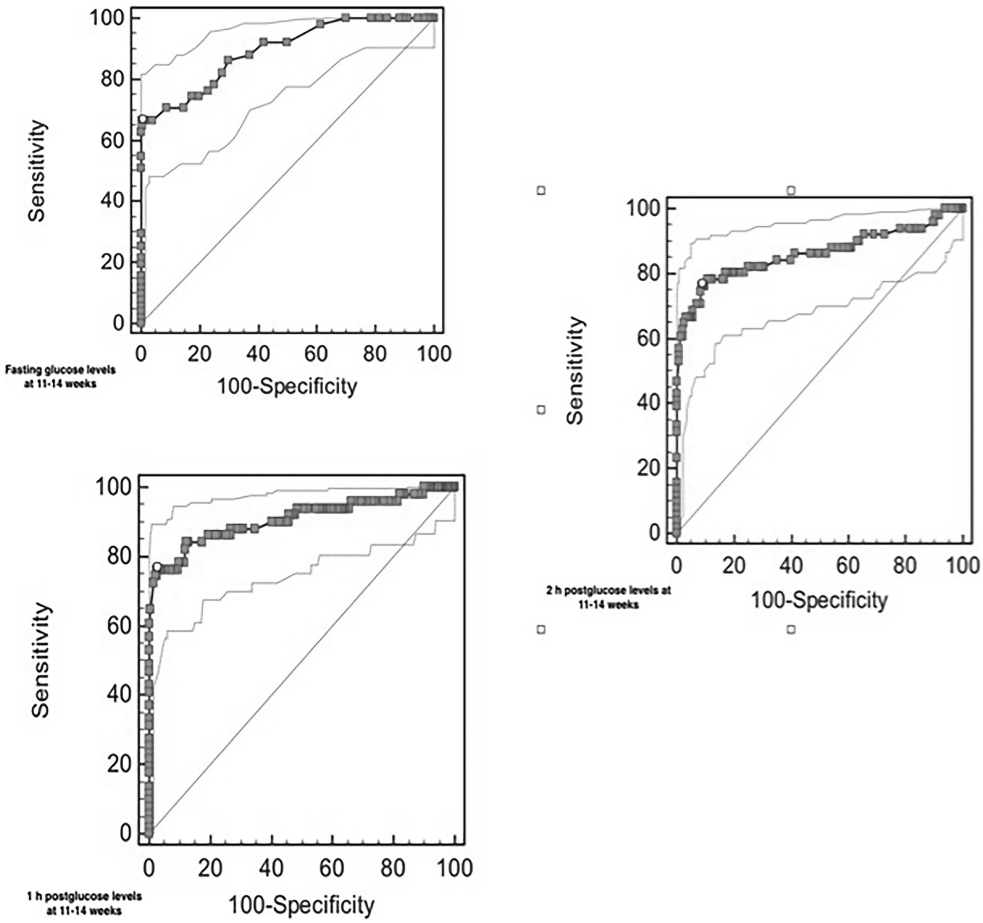

Fig. 2 ROC curves in the prediction of GDM by the 75-g OGTT at 11–14 weeks gestation

time. Consistent with the results of this study, Siegmund et al. [11] evaluated the daily blood glucose profiles of healthy, normal-weight pregnant women at different gestational stages and demonstrated a tendency towards increased blood glucose levels during gestation weeks 16, 22, and 30. The widely accepted timing for GDM screening of 24–28 weeks gestation is based on achieving the maximum GDM detection rate by testing as late in pregnancy as possible while still allowing for an optimal therapy duration to reduce or prevent GDM-associated maternal and perinatal complications.

Plasencia et al. [12] examined the performance of the cutoff values established for GDM screening and diagnostic tests in

Table 5 Perinatal and neonatal outcomes of the groups

	Normal (n = 299)	Early onset GDM (n = 41)	Late onset GDM (n = 10)
Preeclampsia	2 (0.6)[a]	0 (0)[a]	0 (0)[a]
Preterm birth <37 week	31 (10.3)[a]	3 (7.3)[a]	1 (10.0)[a]
Gestational age at delivery	39.1 (34.5–41.0)[a]	38.5 (33.1–41.2)[a]	39.3 (35.5–40.1)[a]
Delivery by cesarean section	123 (41.1)[a]	18 (43.9)[a]	5 (50.0)[a]
Birth weight (g)	3255 (1850–4650)[a]	3540 (2280–4750)[b]	3320 (2650–4560)[a]
Macrosomia (>4500 g)	8 (2.6)[a]	2 (4.8)[a]	1 (10)[a]
1-min Apgar score < 7 (n)	8 (2.6)[a]	2 (4.8)[a]	0 (0)[a]
5-min Apgar score < 7 (n)	7 (2.3)[a]	1 (2.4)[a]	0 (0)[a]
Neonatal hypoglycemia	7 (2.3)[a]	4 (9.7)[a]	1 (10)[a]
Neonatal intensive care unit requirement	10 (3.3)[a]	2 (4.8)[a]	0 (0)[a]

Each subscript letter denotes a subset of group categories whose row proportions do not differ significantly from each other at the 0.05 level

1765 patients with singleton pregnancies during their first trimester. Their results suggested that the 1-h plasma glucose cutoff level after a 50-g glucose load should be 130 mg/dl rather than 140 mg/dl. The authors recommended reducing the cutoff levels for a positive 100-g OGTT from 190 to 161 mg/dl, from 165 to 128 mg/dl, and from 145 to 107 mg/dl for the 1-, 2-, and 3-h post-challenge measurements, respectively. In our study, there were no remarkable changes in the FPG cutoff value for a first-trimester diagnosis of GDM. However, for the 1- and 2-h post-glucose loads following a 75-g OGTT, reductions in the cutoffs from 180 to 173 mg/dl and from 153 to 129 mg/dl, respectively, were recommended, as these new values would allow GDM diagnosis as early as the first, rather than the second, trimester of pregnancy. Lowering the cutoffs for both time points improved the sensitivity of the 75-g OGTT but at the expense of reducing the specificity.

In conclusion, in this study, we demonstrated that the 75-g OGTT based on the one-step approach of the IADPSG was successful in detecting 80.3% of GDM cases as early as the first trimester of pregnancy. To increase the diagnostic performance of the test during the first trimester, the currently used cutoffs should be lowered to enable early detection of GDM. The detection of GDM during the first trimester would help reduce the adverse effects of hyperglycemia by allowing preventative measures to be initiated at an earlier stage of pregnancy. A further benefit would be avoiding unnecessary testing during the second trimester of pregnancy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):26–35
DOI 10.1007/s13410-017-0553-6

ORIGINAL ARTICLE

Lifestyle interventions for gestational diabetes mellitus to control blood glucose: a meta-analysis of randomized studies

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Received: 22 November 2016 / Accepted: 21 February 2017 / Published online: 9 March 2017

Abstract The purpose of this study was to study the effect of lifestyle intervention on the blood glucose control of women with gestational diabetes mellitus (GDM). A meta-analysis was conducted by including randomized controlled trials that compare any form of lifestyle intervention with the usual care. A search of PubMed, MEDLINE, Elsevier Science Direct, Springer, and Web of Science for studies published from 2000 to 2016 was undertaken to identify relevant studies. Methodological quality of the studies was assessed according to the Jadad scale. Random-effort models were used to analyze the weighted mean difference and its 95% confidence interval (CI). From 776 studies, 7 randomized controlled trials (RCTs) (3685 women) were selected for meta-analysis based on our inclusion and exclusion criteria. The evidence suggests that lifestyle, dietary, and activity intervention for women with GDM lowers the fasting plasma glucose (FPG) and 2-h postprandial glucose (2hPG). The reduction in FPG was observed to be statistically significant in the meta-analysis (4 RCTs; n = 1086; −0.16 [95% CI, −0.31 to −0.01]). The reduction in 2hPG was also observed to be statistically significant in the meta-analysis (3 RCTs; n = 849; −0.32 [95% CI, −0.61 to −0.03]). However, no effects of lifestyle interventions were identified in women with GDM in relation to body mass index and hemoglobin A1c. This meta-analysis provides further evidence that lifestyle intervention is associated with reducing FPG and 2hPG in women with GDM.

Keywords Gestational diabetes mellitus · Lifestyle interventions · Meta-analysis

Introduction

An increase in the number of mothers entering pregnancy as obese and with advancing maternal age has contributed to this escalation in rates of gestational diabetes mellitus (GDM). Women with GDM usually improve their insulin resistance following the baby's birth, resolving their GDM [1]. However, about half of mothers with GDM are expected to develop type 2 diabetes within 5 years after pregnancy [2]. For the offspring, GDM in their mother is a major contributor to obesity and type 2 diabetes in later life [3].

GDM is defined as the diabetes diagnosed during pregnancy that is not clearly overt diabetes. [4] According to this definition, pregnant women with glycemic levels that meet the thresholds of overt diabetes are considered to have pre-existing diabetes and the rest are diagnosed as GDM. [5] There was much controversy in the past about the association of mild hyperglycemia with adverse pregnancy outcomes, which was resolved by the landmark observational study by Metzger et al. [6]. GDM is a state restricted to pregnant women whose impaired glucose tolerance is discovered during pregnancy. Women with GDM cannot compensate for the insulin resistance, which is produced by a combination of hormonal and inflammatory changes during pregnancy [7]. Women with GDM and their children are at risk of adverse outcomes in pregnancy and in the long term [8].

Electronic supplementary material The online version of this article (doi:10.1007/s13410-017-0553-6) contains supplementary material, which is available to authorized users.

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Lifestyle interventions involving exercise and a healthy diet in high-risk adults have been found to reduce progression to type 2 diabetes by more than 50% [9–12]. However, little attention has been given to the potential benefits of such strategies in women with GDM. There is a need for safe, simple, effective, and acceptable interventions that prevent the development of GDM. Such an approach has the potential to improve maternal and child health, with significant savings for the health care system [13]. Therefore, the goal of this meta-analysis is to provide researchers and practitioners with a comprehensive overview of the randomized controlled trials of lifestyle interventions that are designed to control blood glucose among women with GDM.

Methods

This meta-analysis was performed according to the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis statement [14].

Literature search

We conducted a detailed search using the electronic databases PubMed, MEDLINE, Elsevier Science Direct, Springer, and Web of Science for reports of randomized controlled trials (RCTs) published from 2000 to 2016 without any language restriction. Medical subject heading terms used were gestational diabetes mellitus RCT and intervention.

Study selection

Studies were selected in a two-stage process. First, we screened the titles and abstracts using the pre-specified inclusion criteria for relevant citations. Then, we assessed the full texts of the selected abstracts. If the abstracts did not provide enough information to decide upon inclusion or exclusion, then we extracted the full text for evaluation.

The inclusion criteria were as follows: (1) the population included women with GDM; (2) the study type was RCT, in which the intervention group receives lifestyle changes including diet, physical, or education and in which the control group receives the usual care; and (3) for evaluation outcomes, at least one outcome of interest was reported, such as weight gain, body mass index (BMI), fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), 2-h postprandial glucose (2hPG), and waist circumference.

The exclusion criteria were as follows: (1) women with other types of diabetes; (2) the effect of intervention was not specific (not randomized controlled studies); (3) there was no specific group number; (4) the full text was not available; and (5) the original data of the study could not be converted or used.

Data extraction and quality assessment

We extracted the following data from each included article: (1) characteristics of participants (number, age, and obstetric characteristics); (2) intervention features (type, duration, frequency, and intensity of lifestyle intervention); (3) target of the study; (4) strengths and weakness of each RCT; and (5) results of outcomes for FPG, BMI, HbA1c, 2hPG, weight gain (in kg), and waist circumference (in cm). Risk of bias was evaluated according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis recommendation [14].

The quality assessment was performed using the Jadad scale [15]. This scale includes five items to be assessed as "yes" or "no" depending on whether the clinical trials met quality criteria in the following areas: sequence generation, allocation concealment, blinding, and incomplete outcome data.

Data synthesis and analysis

Statistical analysis was carried out by using Stata Software. Random-effect model was used to analyze the weighted mean difference (WMD) and its 95% confidence interval (CI). WMDs and 95% CIs were calculated for weight gain, BMI, FPG, and so on [16]. The random-effect model was selected to summarize the pooled WMD. Cochran's Q-statistic was applied to assess the heterogeneity of the studies [17]. The percentage of total variation across the studies as a result of heterogeneity was determined by using I^2. The magnitude of the heterogeneity or inconsistency was classified as follows: (1) small, $I^2 < 25\%$; (2) medium, $I^2 = 25–50\%$; and (3) large, $I^2 > 50\%$ [18]. The sensitivity analysis was assessed, deleting each study from the model, and the pooled analyses were conducted without this study.

Publication bias was evaluated by using funnel plot and Eggers' test if five or more than five studies were included for a particular outcome [19].

Results

Study selection

Figure 1 shows the study selection process. Our literature search identified 776 studies initially. After using the literature management software EndNoteX7 to eliminate duplicates, 620 articles remained. Of these, 527 were excluded after reviewing the titles and abstracts. The remaining 93 were evaluated and selected for a more detailed evaluation of the full article. Of those, 86 studies were excluded, because they did not meet the inclusion criteria: 11 were not RCTs, 11 included interventions that did not meet standards, 30 included participants who did not meet the inclusion criteria, 9 did

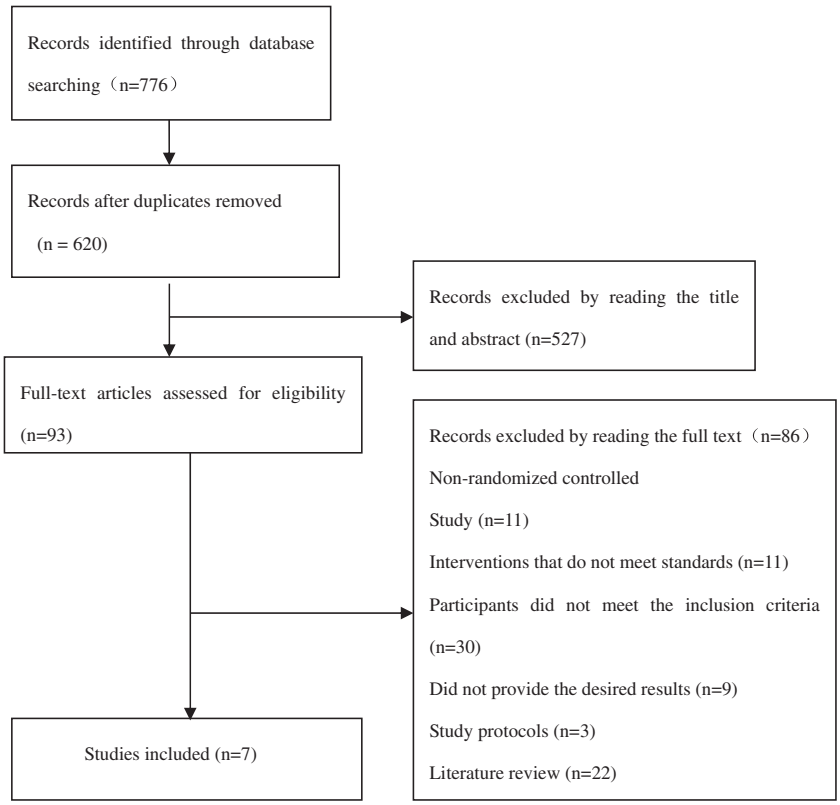

Fig. 1 Flow diagram of the study selection process

not provide the desired results, 3 were study protocols, and 22 were literature reviews. Finally, 7 articles about RCTs with 3685 women were included in the meta-analysis [20–26].

Study characteristics

Table 1 summarizes the study characteristics. The studies were gathered from several countries: one in the United Arab Emirates [20]; three in China, but in different health centers or hospitals [21, 22, 26]; one in Spain [23]; one in Thailand [24]; and one in England [25]. In all of them, women in the control group received the usual care.

The description of interventions in the included studies is shown in Table 2. Of the seven included studies, one study used dietary intervention only and self-monitoring of glucose levels [25]; six studies used a combination of diet and lifestyle including physical activity. Lifestyle interventions included diet and exercise, personal guidance on health education, and regular supervision.

Quality assessment

All seven studies included in the review reported the baseline condition of the patients, and all mentioned "random". Sequence generation was performed and described adequately in two studies [22, 25]. The sequence generation method for the other studies was unclear. Allocation was properly concealed in three studies [22, 24, 25].

All trials had incomplete outcome data, and patients were either excluded or lost to follow-up in both the intervention and the control groups. Four studies described the reason as missing data or as lost to follow-up [20, 23–25]. There was no other apparent risk of bias in any of the included studies. Three RCTs were judged to be of medium quality [22, 24, 25]. The rest were deemed low quality.

Synthesis of results

Table 3 and Figs. 2 and 3 display the results of all of the included studies in both comparison groups.

For BMI, there are 4 studies [21, 23, 25, 26] with 1874 subjects. There is obvious heterogeneity in the result (I^2 = 86.4%, P < 0.01). Using a random-effect model for the analysis, WMD = −0.34 (−1.08 to 0.41). Then, using the Z test, Z = 0.89, P = 0.38, which suggests that there is no statistically significant difference in BMI between the lifestyle intervention group and the control group.

For FPG, there are 4 studies [21, 23, 24, 26] with 1086 subjects. There is obvious heterogeneity in the result (I^2 = 78.1%, P < 0.01). Using a random-effect model for the analysis, WMD = −0.16 (−0.31 to −0.01). Then, using the Z test, Z = 2.12, P = 0.03, which suggests that there is a

Table 1 Characteristics of the included studies

Author (year)	Ethnic group country	Participants	Sample size	Intervention	Outcome measure(s)
Elnour et al. 2008 [20]	United Arab Emirates	Patients diagnosed with GDM	165 99 (intervention arm) and 66 (control arm)	A structured pharmaceutical care service including education and introduction of intensive self-monitoring	plasma glucose and HbA1c; maternal complication; neonatal complication etc.
Hu et al. 2012 [21]	China	Diagnosed with GDM from 2005 to 2009	1180 586 (intervention arm) and 594 (control arm)	Dietary intervention; physical activity intervention and monitoring	Body weight; BMI; waist circumference; FPG 2hPG; HbA1c; body fat etc.
Yang et al. 2014 [22]	China	Women with GDM but without diabetes at 26.3 (interquartile range: 25.4–27.3)gestational weeks	700 339 (intervention arm) and 361 (control arm)	An individualized dietary advice and physical activity counseling	Weight gain; BMI; HbA1c; macrosomia; 1hPG; 2hPG etc.
Perez-Ferre et al. 2014 [23]	Caucasian 63.4%; Aispanic 13.4%; other 23.2% Spain	Women diagnosed with GDM between 24 and 28 weeks of gestational	237 126 (intervention arm) and 111 (control arm)	An educational program on nutrition and a monitored physical activity program	The rate of glucose disorders; BMI; HbA1c; FPG; HDL; LDL etc.
Youngwanichsetha et al. 2014 [24]	Buddhist 56.5%; Islam 43.5% Thailand	Pregnant women with GDM	170 85 (intervention arm) and 85 (control arm)	Mindful eating and yoga exercise	FPG: 2hPG; HbA1c etc.
Landon et al. 2009 [25]	Black 11.5%;White 25.3%; Asian 5.2%;Hispanic 57.0% other 1.0% England	24th to 31st week of gestation met the criteria for mild GDM	958 485 (intervention arm) and 473 (control arm)	Dietary intervention; self-monitoring	BMI; weight gain pre-eclampsia etc.
Cao et al. 2012 [26]	China	Pregnant women with GDM	275 127 (intervention arm) and 148 (control arm)	Individualized diabetes education, dietary exercise advice and self-monitoring glucose	Macrosomia; hypoglycemia; HDL; LDL; BMI; FPG; 2hPG etc.

GDM gestational diabetes mellitus, *BMI* body mass index, *FPG* fasting plasma glucose, *HbA1c* hemoglobin A1c, *2hPG* 2-h postprandial glucose, *OGTT* oral glucose tolerance test, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *HOMA-IR* homeostasis model assessment of insulin resistance

Table 2 Description of interventions in the included studies

Author (year)	Intervention
Elnour et al. 2008 [20]	Pharmaceutical care interventions: The clinical pharmacist educated each patient, in a structured fashion, on GDM and its management. This included providing advice on: diet, exercise, keeping blood sugar levels within normal range, recommended timing and frequency of self-monitoring of plasma glucose, how to record plasma glucose results in diary cards. Subjects were instructed to measure plasma glucose at least five times daily (fasting, 1 and 2 h post-meal, 2–6 PM and prior to bed time) for 3–4 days per week and to record readings in a diary card which was supplied to them.
Hu et al. 2012 [21]	Dietary intervention (weeks 1–4): six goals of the intervention, including: (1) reduction in 5–10% of initial body weight in women with BMI 24 kg/m^2 through the reduction of at least 10% of total calories of their normal meals, and no weight loss for GDM women with BMI < 24 kg/m^2; (2) total fat intake <30% of energy consumed; (3) saturated fat intake <10% of energy consumed; (4) carbohydrate intake 55–65% of energy consumed; (5) fiber intake 20–30 g/day; and (6) moderate or vigorous exercise for at least 30 min daily, 7 days each week. Physical activity intervention (weeks 1–4): Participants will be instructed to engage in moderate or vigorous physical activity during commuting (walking or cycling to/from work) or leisure time (e.g., walking, bicycling, etc.) for at least 15 min/day, 7 days/week during week 1. Diet and physical activity monitoring (week 4 to month 12): Each subject will complete a questionnaire on changes in major dietary habits and physical activity habits from the last visit, and 3-day 24-h food records five times during the first year for assessment by the dietitian. The dietitian will review questionnaires and food records, calculate the nutrient intakes via a dietary analysis software developed by China CDC, provide an assessment about deviations from the suggested diets and exercises, and then offer specific suggestions at each visit. Body weight will be measured at each visit to monitor compliance.
Yang et al. 2014 [22]	The additional intervention of SC was delivered by an intervention team from TWCHC, consisting of trained nurses and doctors. All women in the SC group were offered an individualized dietary advice and physical activity counseling at entry to the trial. Different energy intakes were recommended based on pre-pregnacy body mass index (BMI) classification for Chinese adults. All women were asked to be engaged in at least 30 min of light to moderate physical activity daily, i.e., walking. All women were offered a free glucose meter with memory function and free test strips. They were asked to perform SMBG, 4 times a day for the initial 2 weeks and then daily at different time points in rotation (pre-breakfast and 2 h after three meals).
Perez-Ferre et al. 2014 [23]	Nutritional recommendations: high consumption of fruits and vegetables (five or more serving a day), high consumption of legumes (more than two serving per week), high consumption of nuts (more than three serving a week), daily use of virgin olive oil (more than 40 cc per day), at least three serving per week of oily fish, low consumption of red and processed meats (less than two serving per week) and low consumption of non-skimmed dairy products (less than two serving per week). Physical activity program: The exercises were performed during sessions of 50–60 min 4 days per week (2 days at the hospital and 2 days at home). The exercise sequence was as follows: warm-up exercises (10 min), aerobic activity; cycle ergometer and treadmill (15 min); muscular strengthening exercises, biceps, triceps, abdominals, quadriceps, with two to three sets of eight to ten repetitions for each muscular group (15 min); resting periods between the sets (10 min); and relaxation exercises (5 min).
Youngwanichsetha et al. 2014 [24]	Mindfulness eating for pregnant women with GDM was composed of five steps: (1) setting a goal for blood glucose control, (2) integrating medical nutrition therapy including carbohydrate choices and low glycemic index food, (3) considering portion size, (4) being aware while consuming diabetic food, and (5) eating slowly for 30 to 45 min. Yoga exercise: It was designed for 15–20 min daily practice for 5 days per week for 8 weeks. Each posture should be repeated for ten times. Yoga asanas for pregnant women comprised 9 postures namely (1) padmasana, (2) brahma mudra, (3) shoulder circling, (4) modified parvatasana, (5) modified gomukhasana, (6) modified tadasana, (7) modified chakrasana, (8) modified bharadvajasana, and (9) dandasana
Landon et al. 2009 [25]	Treatment group: dietary intervention, self-monitoring of blood glucose, and insulin therapy, if necessary
Cao et al. 2012 [26]	Comprehensive intensive therapy: included individualized diabetes education, dietary and exercise advice, and instructions on how to self-monitor glucose levels. The patients in this group were asked to perform self-monitoring four times per day until their glucose levels remained in the recommended range for 2 weeks (fasting blood glucose levels, >4.0 and ≤5.5 mmol/L; 2-h postprandial levels, <6.7 mmol/L). In addition, the patients were regularly monitored by a diabetes physician in the Endocrinology Clinic and received careful adjusting of diet and therapy at least every 2 weeks.

statistically significant difference in FPG between the lifestyle intervention group and the control group (Fig. 2).

For 2hPG, there are 3 studies [21, 24, 26] with 849 subjects. There is obvious heterogeneity in the result (I^2 = 59.6%, P = 0.08). Using a random-effect model for the analysis, WMD = −0.32 (−0.61 to −0.03). Then, using the Z test, Z = 2.16, P = 0.03, which suggests that there is a statistically significant difference in 2hPG between the lifestyle intervention group and the control group (Fig. 3).

For HbA1c, there are 4 studies [20–23] with 1506 subjects. There is obvious heterogeneity in the result (I^2 = 81.0%, P < 0.01). By using a random-effect model for the analysis, WMD = 0.00 (−0.11 to 0.10). Then, using the Z test, Z = 0.12, P = 0.90, which suggests that there is no statistically

Table 3 Effect estimates for randomized controlled trials of lifestyle changes versus usual care

Outcome or subgroup	Participants	Studies	Statistical method	Effect estimate
BMI	1874	4	WMD (fixed, 95% CI)	−0.34 (−1.08, 0.41)
FPG	1086	4	WMD (fixed, 95% CI)	−0.16 (−0.31, −0.01)
2hPG	849	3	WMD (fixed, 95% CI)	−0.32 (−0.61, −0.03)
HbA1c	1506	4	WMD (fixed, 95% CI)	0.00 (−0.11, 0.10)

WMD weighted mean difference, *BMI* body mass index, *FPG* fasting plasma glucose, *HbA1c* hemoglobin A1c, *2hPG* 2-h postprandial glucose

significant difference in HbA1c between the lifestyle intervention group and the control group.

Regarding sensitivity analysis, when each study was deleted from the model once, the results remained significant across all of the analyses.

There was no significant publication bias, as evidenced by the Begg's funnel plot. Results for the Begg test and Egger test, respectively, were as follows: BMI (P = 1.00and P = 0.87); FPG (P = 0.31 and P = 0.18); 2hPG (P = 0.30 and P = 0.05); and HbA1c (P = 0.30 and P = 0.26).

Discussion

Summary of main findings

Lifestyle, dietary, and activity intervention for women with GDM lowers FPG and 2hPG. The reduction in FPG was observed to be statistically significant in the meta-analysis (4 RCTs; n = 1086; −0.16 [95% CI, −0.31 to −0.01]). The reduction in 2hPG was also observed to be statistically significant in the meta-analysis (3 RCTs; n = 849; −0.32 [95% CI, −0.61 to −0.03]). No effects of lifestyle interventions were identified in women with GDM in relation to BMI and HbA1c.

Interpretation

Treatment of GDM remained controversial mostly due to the lack of a uniform standard for defining glucose intolerance during pregnancy [27]. Because of this reason, individual studies on the topic produced varying results causing confusion about efficacy and safety of GDM treatment.

Previous reviews have attempted to make conclusions regarding specific effective components of interventions. Suggestions include weight monitoring, and setting weight goals could be useful [28], as well as monitoring along with education counseling and physical activity [29, 30]. Another review suggested that interventions should be based on the Theory of Planned Behavior, but the rationale for using this model over others in this population was unclear [31]. A more recent review by Gardner et al. assessed interventions targeting gestational weight gain from a psychological perspective and specifically examined intervention content and delivery methods [32]. Only two of the studies reported basing

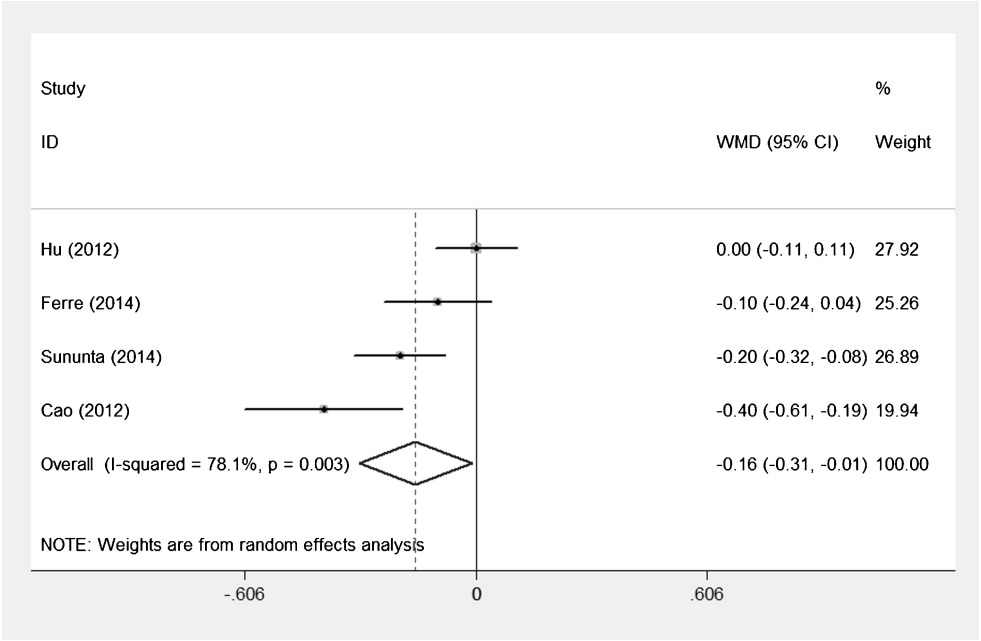

Fig. 2 Forest plot of weight gain in intervention group versus control group

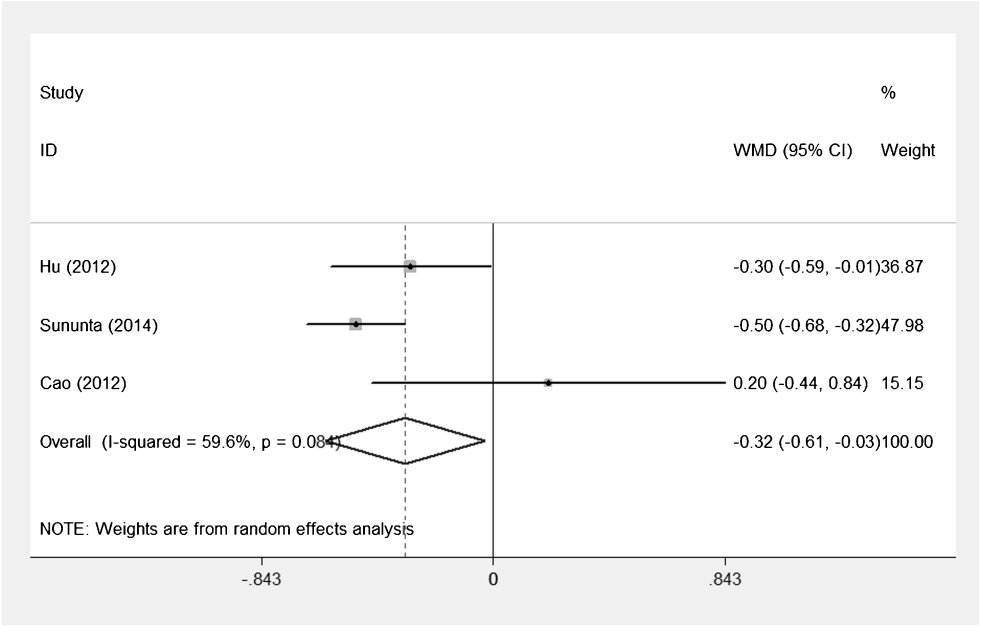

Fig. 3 Forest plot of 2hPG in intervention group versus control group

interventions on theory. The studies used, on average, five behavior change strategies (self-monitoring, feedback provision, and setting behavioral goals were the most common); however, no conclusions could be drawn as to their contribution to study outcomes. A review by Whitelaw et al. [33] pointed out that increasing activity levels and exercise helps to control GDM. Thirty minutes per day of physical activity is suggested, and walking, swimming, or yoga is suggested as the best types of exercise in pregnancy. Women with GDM should be given a home blood glucose monitoring meter and taught how to use it. They are normally advised to monitor at least four times per day. The recommended times to test are as follows: on waking in the morning and then 1 h after the first bite of each meal.

None of these reviews examined intervention components systematically. In this sense, our meta-analysis includes some recently published well-designed RCTs with a larger number of participants in which lifestyle interventions included dietary intervention, physical activity intervention, and self-monitoring glucose. Our study uses a growing body of evidence that aims to evaluate lifestyle interventions as a means to minimize the adverse outcome associated with GDM in pregnancy. Based on the data from these studies, results of our analysis indicated that treating GDM women with lifestyle interventions may reduce FPG and 2hPG. These results could be linked with the fact that physical exercise in these programs was initiated before the normal increase in insulin resistance that occurs with advancing gestation [34], so that chronic changes in the regulation of skeletal muscle glucose uptake had already been adopted when this increased resistance was reached; therefore, women might have better conditions to handle the metabolic stress of pregnancy [35]. Diet-based interventions appear to show some benefits for pregnant women with GDM. This could be due to the following reasons: individual dietary and components, change in gestational weight gain, and effect of nutritional supplements. The interventions promoted the intake of healthy components such as fiber and food rich in vitamins that may have an additive effect in reducing the concentrations of maternal glucose [21, 23]. The women in the intervention group had reduced total energy intake and glycemic load compared to the control group [21, 24]. Low glycemic index diet reduces the increase in insulin resistance observed in pregnancy, thereby reducing the risk of GDM [36]. The risk of GDM is known to be reduced by a quarter with each 10 g/day increment in total fiber intake [37]. The largest benefit with diet was observed where there was a multidisciplinary input into the intervention, with the use of food diaries [20] and feedback methods. Women in the intervention vs control group, especially among those who were overweight at baseline, have reduced their body weight and other outcome measurements of obesity, such as BMI, body fat, and waist circumference [21]. Sununta et al. [24] carried out an RCT to investigate the effects of mindfulness eating and yoga exercise on blood sugar levels of pregnant women with gestational diabetes mellitus. They found that the intervention group showed significantly reduced FPG, 2hPG, and HbA1c in the intervention group ($P < 0.05$). So, it should be recommended in diabetes self-management.

A meta-analysis [38] evaluates the effect of medical nutritional intervention on clinical outcome of GDM. The effect of medical nutritional intervention was described in terms of fasting blood glucose, birth body mass, and incidences of macrosomia, cesarean section, postpartum glucose intolerance, and neonatal long-term chronic disease.

A systematic review [39] evaluates the effect of telemedicine on GDM service and maternal and fetal outcomes, then found that telemedicine was associated with significantly fewer unscheduled GDM clinic visits, quality of life, glycemic control (HbA1c, pre- and postprandial blood glucose level), and cesarean section rate that were similar between the telemedicine and usual care groups. Olah [40] conducted on educational and intervention programs for GDM management. Their conclusion is as follows: interventions that include adopting a low glycemic index diet and increasing levels of activity appear to be successful at reducing maternal blood glucose levels and reducing insulin requirements during pregnancy. Reducing maternal blood glucose levels, in turn, is associated with a reduction of macrosomia and maternal weight gain.

Russo [41] carried out a meta-analysis suggested that physical activity in pregnancy provides a slight protective effect against the development of GDM. Taber [42] conducted a literature search of RCTs of lifestyle interventions among women with a history of GDM. Preliminary findings suggest that such interventions can improve diabetes risk factors in women with a history of GDM.

In Elnour et al. [20] study, intervention patients received a structured pharmaceutical care service (including education and introduction of intensive self-monitoring) while control patients received traditional services. Statistically significant improvements were shown in the intervention group for knowledge of diabetes, health-related quality of life, control of plasma glucose and HbA1c, maternal complications, and neonatal complications. Ferre et al. [23] concluded that lifestyle intervention was effective for the prevention of glucose disorders in women with prior GDM. Also, body weight gain and an unhealthy fat intake pattern were found to be the most predictive factors for the development of glucose disorders. Patients after dietary intervention and self-monitoring of blood glucose have reduced the risks of fetal overgrowth, shoulder dystocia, cesarean delivery, and hypertensive disorders [25].

There was also wide variation in the types of interventions evaluated in the studies. The majority used lifestyle interventions, and most provided generic guidance comprising mainly dietary advice, physical activity counseling information, and self-monitoring of glucose. There was considerable heterogeneity in the intervention designs and no obvious patterns between intervention type and study outcomes. The individual patient characteristics may also play an important role in achieving glycemic goals with a specific intervention.

Strengths and limitations

To date, this study is the most updated review of RCTs conducted on this topic. Our results are more precise than those of the earlier reviews [43, 44]. Our findings were limited by differences in the inclusion criteria among the studies; by variations in the components of the interventions, such as duration, intensity, and frequency; by non-standardized care in the control group; and by non-uniform definitions of GDM. Furthermore, women in the intervention groups had more than one intervention, such as diet and activity, making it difficult to delineate the beneficial effect of an individual intervention. It is possible that a different criterion for the diagnosis of GDM may have yielded changed estimates of effect [45]. We could not achieve sufficient power to report significant results for some important outcomes like perinatal or neonatal mortality, preterm birth, small for gestational age infants, cesarean section, and pre-eclampsia. Lack of blinding in the studies may have affected the validity of data.

Conclusions

This study shows that lifestyle intervention is associated with reducing FPG and 2hPG in women with GDM. No effects of lifestyle interventions were identified in women with GDM in relation to BMI and HbA1c.

Acknowledgements My deepest gratitude goes first and foremost to Professor Xia Wang, my supervisor, for her constant encouragement and guidance. She has walked me through all the stages of the writing of this paper. Without her consistent and illuminating instruction, this thesis could not have reached its present form. I also owe my sincere gratitude to my friends Baihui Zhang who gave me their help and time in listening to me and helping me work out my problems during the difficult course of the thesis.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals (1) Statement of human rights

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

(2) Statement on the welfare of animals

This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):36–41
DOI 10.1007/s13410-017-0557-2

ORIGINAL ARTICLE

Low vitamin D and risk for small for gestation age babies in gestational diabetes

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Received: 21 December 2016 / Accepted: 5 April 2017 / Published online: 26 April 2017

Abstract This study aims to find the possible correlation of vitamin D with the onset of gestational diabetes mellitus (GDM) and its effect on fetal growth. A case-control study was conducted recruiting pregnant women in their second trimester. All subjects were classified as per the guidelines of the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criterion. Fetal growth scans were recorded at 28th week of gestation. Venous blood was collected and serum levels of 25-(OH) D, Calcium, TNF alpha were measured. Vitamin D concentration in cases was lower (2.40 ± 1.10 ng/ml) as compared to controls (5.50 ± 1.74 ng/ml), while serum calcium levels of both groups were under normal range. TNF alpha level in cases was higher as compared to controls ($p < 0.001$). On ultrasound scan, 47% of the babies of GDM mothers were small for gestational age. No difference was observed in terms of femur length and fetal weight in both groups ($p > 0.05$). Weak negative correlation of vitamin D with insulin resistance ($r = -0.304$; $p = 0.004$), positive correlation with fetal growth ($r = 0.303$; $p = 0.043$) and a strong negative correlation with TNF alpha levels ($r = -0.703$; $p = 0.023$) were observed. Low vitamin D levels may be associated with the onset of gestational diabetes and affect fetal growth and development. Hence, antenatal screening and timely intervention is recommended.

Keywords GDM · Vitamin D · Small for gestational age

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Introduction

Vitamin D is a fat-soluble secosteroid hormone. It is responsible for maintaining calcium homeostasis and enhancing bone mineralization [1]. The active form of vitamin D is generated first by 25α-hydroxylase to produce 25 hydroxyvitamin D [25(OH) D] or calcidiol in the liver, followed by the second hydroxylation step in the kidney, mediated by 1α-hydroxylase, to produce 1,25- dihydroxycholecalciferol (1,25-$(OH)_2$D) or calcitriol [2]. Although its metabolic role in skeletal homeostasis is clear, there has been an ongoing debate over the role of vitamin D in preventing various disorders such as cardiovascular diseases and type 2 diabetes mellitus (T2DM) [3]. In the previous years, several large observational studies have suggested an association between vitamin D deficiency and the onset of type 2 diabetes [4]. However, the physiological role of vitamin D in pregnancy and other pregnancy associated complications such as gestational diabetes mellitus (GDM) is still not fully understood. Furthermore, the results reported are not consistent and vary according to different ethnic groups and form of vitamin D tested (Table 1).

GDM is a growing global concern, with prevalence estimated to be 15–20% [5]. Recent studies conducted in Pakistan showed the incidence of GDM 14.5% [6]. This increasing trend is alarming due to the fact that GDM can lead to a number of adverse consequences for both the mother and the child. These include an increased risk for developing T2DM after the pregnancy for the mother and a predisposition to obesity, and metabolic syndrome later in life for the child [7]. Subsequently, a large proportion of our population is also deficient in vitamin D, which may be a contributing factor towards the high incidence of GDM. It is estimated that 92% ambulatory adults [8] and 84.3% asymptomatic individuals [9] are vitamin D deficient.

Table 1 Summary of relevant recent articles

Year	Author	Country	Type of study	GDM criteria	Key findings
2012	Wang et al. [34]	China	Nested-case-control	ADA	Vitamin D deficiency higher in GDM group
2013	Parildar et al. [20]	Turkey	Case-control	IADPSG	Lower serum vitamin D levels in GDM No association between vitamin D levels, FPG, or INS
2013	Zuhur et al. [19]	Turkey	Cross-sectional	IADPSG	Only women severely deficient in Vitamin D had increased risk of GDM
2013	1. Asemi et al. [35]	Iran	Randomized, double-blind, placebo-controlled clinical trial	WHO	Vitamin D supplementation increased insulin sensitivity, and reduced FPG compared with placebo
2014	Lacroix et al. [23]	Canada	Cross-sectional	IADPSG	Lower first trimester vitamin D showed greater risk of GDM
2014	McManus et al. [24]	Canada	Case-control	CDA	GDM women had significantly lower concentration of vitamin D
2014	Lithy et al. [13]	Egypt	Cross-sectional	WHO	Negative correlation between the glycemic control and vitamin D levels
2014	Whitela et al. [36]	UK	Cross-sectional		No association between vitamin D status and glucose tolerance in pregnancy
2015	Arnold et al. [25]	USA	Nested-case-control	ADA	25[OH]D3 concentrations, but not total 25[OH]D concentrations, were significantly associated with GDM risk
2015	Loy et al. [37]	Singapore	Cohort	WHO	Maternal 25 (OH)D was associated with higher FPG. No relation between 2 h post-load glucose concentration and GDM
2015	Pleskačová et al. [38]	Czech Republic	Case control	WHO	No difference in vitamin D levels of pregnant women with and without GDM. Both groups exhibited high prevalence of vitamin D deficiency
2015	Rodriguez et al. [39]	Spain	Prospective cohort study	WHO	No association of vitamin D levels and the risk of GDM
2016	Haidari et al. [40]	Iran	Cross-sectional study	IADPSG	Serum 25 (OH) D levels were significantly lower in the GDM group
2016	Josefson et al. [41]	North America	Epidemiological study	HAPO	No correlation of maternal serum 25 (OH) D with maternal FPG, insulin sensitivity, and presence of GDM

ADA American Diabetes Association, *IADPSG* International Association of the Diabetes and Pregnancy Study Groups, *CDA* Canadian Diabetes Association, *WHO* World Health Organization, *FPG* fasting plasma glucose, *INS* insulin

During pregnancy, mobilization of maternal calcium increases to meet the demands of adequate fetal bone mineralization. As a consequence, a number of physiological adaptations take place, including increased maternal serum calcitriol, vitamin D binding protein (DBP), placental vitamin D receptor (VDR), and renal and placental CYP27B1 activity to maintain normal serum levels of 25(OH)D and calcium [10]. Maternal 25(OH) D then crosses the placenta and is the main

form of vitamin D for the fetus. Thus, the most important contribution of vitamin D during pregnancy is to escalate calcium absorption and placental calcium transport [11] (Fig. 1). Additionally, vitamin D also regulates immune system and inhibits inflammation by restraining inflammatory cytokines including TNF-α in the placenta [10]. Vitamin D plays a role in glucose homeostasis during pregnancy by multiple mechanisms. The release of insulin from the ß cells of the pancreas requires calcium, and vitamin D is required for the uptake of calcium, as it increases calcium channel and calcium binding protein expression in the small intestines [12]. It also improves the sensitivity of the target cells like adipose tissue, liver, and skeletal muscles to insulin [13]. The serum concentration of 25(OH)D is almost 1000-fold of that of 1,25(OH)D, and it has a half-life of 25 days, which infers it as a more stable marker, especially during pregnancy [14]. Furthermore, the American Endocrine Society has labeled 25(OH)D as a better marker for estimating vitamin D deficiency in young children, pregnant women, older persons, and immigrants [15]. Unfortunately, limited and conflicting data on vitamin D deficiency in GDM is available especially in Pakistan. It is therefore pertinent to measure the levels of vitamin D [25(OH) D] and relate them with the occurrence of GDM in an effort to understand whether vitamin D levels play a role in the development of this disease or not. Therefore, the aim of this study is to find the possible correlation of vitamin D with the onset of GDM and fetal growth.

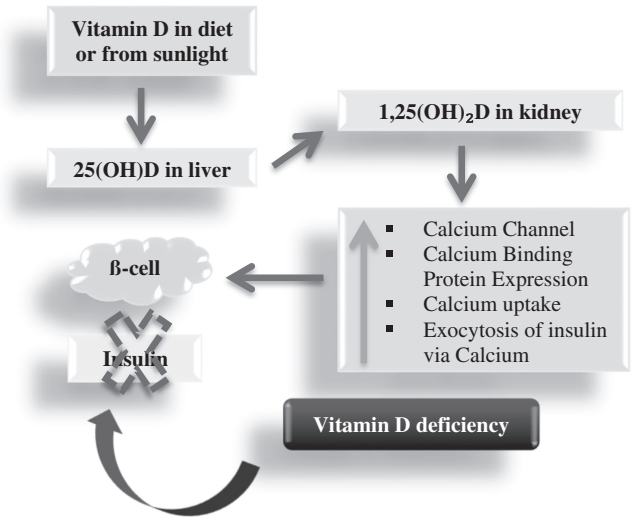

Fig. 1 Vitamin D is synthesized de novo when 7-dehydroxycholestrol in the skin is converted to cholecalciferol (D_3), using sunlight. Vitamin D_3 is then converted to 25-hyroxyvitamin D_3 in the liver and the enzyme used is 25-hydroxylase. In the kidneys, 25 (OH) D_3 is catalyzed by the enzyme 1-hydroxylase and is converted to 1,25-dihydroxyvitamin D_3. Vitamin D exerts its metabolic effect by increasing calcium absorption from the small intestine and by increasing urinary calcium reabsorption from the kidneys. Calcium is then further used for bone mineralization [42] and insulin release from pancreas (ref). A deficiency in vitamin D may decrease insulin release and also cause increase in pro-inflammatory cytokines leading to insulin resistance

Material and methods

A case-control study was conducted from August 2015 till August 2016, recruiting pregnant women in their second trimester of gestation from the Aga Khan University out-patient department. The minimum sample size calculated for this study was $n = 176$ based on the prevalence of GDM as 15% [16] . All study participants gave a verbal and written consent and the study was approved by the institutional ethical committee. All subjects were given a 75 g oral glucose tolerance test (OGTT), as per the guidelines of International Association of Diabetes and Pregnancy Study Groups (IADPSG) criterion. The criteria states that diagnosis of GDM is made when any one of the following plasma glucose readings are observed: a fasting plasma glucose level ≥ 92 mg/dL or postprandial (2 h post glucose load) plasma glucose ≥153 mg/dL or both [17]. Women falling in the abovementioned category were labeled as "Cases" ($n = 88$) while normoglycemic women were labeled as "Control" ($n = 88$) for this study. The exclusion criteria observed were polycystic ovary disease, preexisting diabetes mellitus, hypertension and other chronic diseases, women taking hormonal supplements or anti-inflammatory drugs, vitamin D supplements, calcium supplements, and using sunscreen regularly. Ten milliliters of venous blood was collected at 28th week of gestation. Serum was separated by centrifugation and stored at −80 °C until further analysis. Vitamin D levels (25-(OH) D) were measured using a commercially available ELISA kit (Kit cat number DK0146, Dia Metra-System, Germany). Vitamin D levels were classified as; deficient < 10 ng/ml, insufficient = 10–29 ng/ml, sufficient = 30–100 ng/ml, and toxic = >100 ng/ml [18]. Serum calcium was measured by kit method on Roche automated clinical chemistry analyzer, while TNF alpha was measured by ELISA kit (Kit cat number KAC1751, Thermo Scientific, USA). Biophysical data such as height and weight and fetal ultrasound scans were recorded from patient record cards.

Statistical analysis

The data was analyzed using SPSS (version 21) IBM, Chicago, Illinois, USA. Data was presented as mean ± SD and absolute number with percentage in parenthesis. Student's t test, Pearson chi-square/Fischer's exact tests were applied to compare groups while Pearson correlation was used to assess the relation between vitamin D levels and insulin resistance. In all instances a p value <0.05 was considered significant.

Results

The detailed results of the study are shown in Tables 2 and 3. No difference was seen in the age, weight, BMI, and hemoglobin levels of the study subjects while low insulin levels and high insulin resistance were seen in the cases versus controls ($p < 0.001$). Interestingly, both our study groups were vitamin D deficient but the concentration seen in cases (2.40 ± 1.10) was even lower as compared to the controls (5.50 ± 1.74). Despite the low vitamin D levels, serum calcium levels of both groups were under the normal range. The inflammatory marker TNF alpha in cases was higher as compared to controls ($p < 0.001$). On ultrasound scan, 47% of the babies of GDM mothers were small for gestational age, while 31% for the control group. Interestingly, no difference was observed in terms of femur length and fetal weight in both groups ($p > 0.05$). Table 2 shows a weak negative correlation of vitamin D concentration with insulin resistance ($r = -0.304$; $p = 0.004$), a positive correlation with fetal growth ($r = 0.303$; $p = 0.043$) and a strong negative correlation with TNF alpha levels ($r = -0.703$; $p = 0.023$).

Discussion

In this study, we report that despite low vitamin D levels observed in the overall pregnant women, the lowest levels were seen in women with GDM as compared to non-GDM women. Studies reported a similar trend that only women who were severely deficient in vitamin D were at a greater risk of developing GDM, though they found no correlation between vitamin D levels and the levels of fasting glucose and insulin [19]. Similarly, no relation between vitamin D levels and the risk of GDM was reported in studies conducted in China, Czech Republic and Istanbul [20–22]. Though we report higher insulin resistance in the GDM group compared to the non-GDM women in our study, surprisingly our results also express decreased insulin levels in GDM women as compared to non-GDM women ($p < 0.001$). This could be attributed to waning out of beta cells due to increased demand in pregnancy or decline in insulin secretion due to vitamin D deficiency, which cause disturbance in the extracellular and intracellular calcium pools and inhibit insulin release, especially in a glucose-burdened condition such as GDM. A weak negative correlation was also seen between vitamin D levels and insulin resistance in our study, which is in agreement with other studies [19, 23–25]. Thus, we can contemplate that vitamin D deficiency may play a role in the etiology of gestational diabetes.

Despite the fact that GDM is associated with large for gestational age (LGA) newborns, various studies have reinforced the association of small for gestational age (SGA) babies with vitamin D deficiency in gestational diabetic women [26, 27], which can be related to the lack of bone growth in the presence

Table 2 Clinical and biochemical characteristics of the study population

Variable	Control ($n = 88$) Mean ± SD	Case ($n = 88$) Mean ± SD	p value
Age (year)	25.44 ± 4.64	27.27 ± 5.18	0.053
Weight at booking (kg)	59.26 ± 11.61	61.90 ± 11.98	0.064
BMI (kg/m^2)	23.80 ± 4.65	25.10 ± 5.63	0.069
OGTT-0 h (mg/dl)	80.28 ± 8.33	103.93 ± 11.70	<0.001
OGTT-2 h (mg/dl)	119.53 ± 15.90	176.20 ± 5.53	<0.001
Hemoglobin (g/dl)	10.56 ± 1.18	10.84 ± 1.21	0.212
Insulin (mIU/L)	39.60 ± 9.02	13.01 ± 2.72	0.001
Insulin Resistance (HOMA-IR)	4.84 ± 4.38	6.74 ± 3.87	0.001
Insulin Sensitivity Index	0.32 ± 0.003	0.30 ± 0.004	0.05
Vitamin D (ng/ml)	5.50 ± 1.74	2.40 ± 1.10	0.172
Calcium (mg/dL)	9.81 ± 1.23	10.54 ± 2.11	NS
TNF alpha (pg/ml)	3038.2 ± 663.59	7154.4 ± 451.81	<0.001
Fetal growth scan			
LGA	4 (4.5)	24 (27.2)	0.023
SGA	24 (31.1)	42 (47.8)	
NGA	59 (64.4)	22 (25.0)	
Fetal weight (kg)	2.40 ± 0.08	2.23 ± 0.05	NS
Femur length (cm)	6.80 ± 1.22	5.77 ± 1.14	NS

OGTT oral glucose tolerance test, *LGA* large for gestational age babies, *SGA* small for gestational age babies, *NGA* normal for gestational age babies, *NS* not significant

Table 3 Correlation of vitamin D

Variable	Pearson's correlation (r)	p value
Growth scan	0.303	0.043
Femur length (cm)	−0.250	0.200
Fetal weight (Kg)	0.315	0.111
HOMA-IR	-0.304	0.004
TNFα	−0.703	0.023

HOMA-IR insulin resistance, *TNF α* tumor necrosis factor alpha

of vitamin D deficiency. The overall rate of SGA babies was higher in our study (47% in GDM cases while 31% in non-GDM controls). Moreover, vitamin D levels showed a weak association with fetal growth ($r = 0.303$), though no association or difference was observed in fetal femur length in both groups. The widespread vitamin D deficiency in our study subjects is probably a valid reason for this high rate of SGA in our study population. Interestingly, no difference was seen in the serum calcium level in our study. Similar findings were seen by Holis and group which stated that the changes in vitamin D concentrations during pregnancy are not followed by a concurrent change in serum calcium levels [28]. Although maternal serum calcium levels fall during pregnancy, as a result of decreased serum albumin, the overall ionized calcium levels remain unchanged [29].

Increased inflammatory cytokines have also been linked with insulin resistance, and vitamin D helps in immune regulation, i.e., suppression of pro-inflammatory cytokines, especially during pregnancy and other endocrine disorders [30]. We report increased levels of TNFα in GDM women as compared to the non-GDM women ($p < 0.001$). This may explain the high insulin resistance seen in our study, as TNF alpha is known to block tyrosine kinase activity in adipocytes [31]. Further, we report a strong negative association of TNF alpha levels with vitamin D levels ($r = -0.703$). This suggests that a vitamin D deficient state weakens the cytokine suppressive function of vitamin D and thus the systemic cytokines increase to cause GDM. Observational studies have been recently conducted to study the association of vitamin D with GDM, and vitamin D supplementation has been shown to bring a decline in the inflammatory profile of patients, a reduction in GDM risk by 14% in USA and improved maternal and neonatal vitamin D status in the Pakistani population [25, 32]. On the contrary, it has also been reported that there are no beneficial effects of vitamin D supplementation on the outcome of GDM [33], though the lack of consistent findings calls for large-scale prospective studies to evaluate the exact mechanism of vitamin D and its role in pregnancy-related complications. We were limited in acquiring a larger cohort preferably from other centers and localities. Moreover, neither did we measure the parathyroid hormone levels nor did we note dietary factors in this study. In spite of that, our findings suggest a strong role of vitamin D deficiency in the risk of GDM and small for gestational age babies.

Conclusion

Low vitamin D levels may be associated with the onset of gestational diabetes and affect fetal growth and development. Hence, antenatal screening and timely intervention is recommended.

Acknowledgement The authors would like to thank the Department of Biological and Biomedical Sciences, Aga Khan University, for funding this project. Also we wish to thank the study volunteers and technical staff of the project.

Authors' contribution SSF and FA were involved in the conception and design, analysis, and interpretation of data. MAS and AS were involved in the acquisition, analysis, and interpretation of data. All authors took active part in drafting the article and revising it critically for important intellectual content. All authors approved the final version submitted for publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Funding source Department of Biological and Biomedical Sciences' research module fund.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):42–46
DOI 10.1007/s13410-017-0571-4

ORIGINAL ARTICLE

Implication of soluble transferrin receptor and ferritin ratio in gestational diabetes

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Received: 13 April 2017 / Accepted: 19 June 2017 / Published online: 16 July 2017

Abstract Ferritin provides a good estimate of body iron stores, but loses its significance during systemic inflammation, a defining feature of insulin resistance (IR). On the other hand, serum transferrin receptor protein (sTfR) represents true iron load of body. This study attempts to identify the utility of sTfR/ferritin index in gestational diabetes mellitus (GDM). Eighty-eight pregnant females were recruited and divided into group A (norm-glycemic pregnant) and group B (GDM) according to the IADPSG criteria. Insulin, hemoglobin, serum iron, ferritin, sTfR, and CRP levels were determined. However, HbA1c and sTfR/ferritin ratio were calculated by formulas. Mann Whitney U and Spearman's rank correlation were used, where $p < 0.05$ was considered significant. Results showed no difference between the groups in terms of age, weight, and BMI. IR was significantly high in GDM as compared to non-GDM subjects ($p < 0.001$), however, the insulin levels were found to be low in GDM group. Hemoglobin was comparatively lower in GDM females ($p = 0.212$). Serum levels of sTfR, ferritin, and hs-CRP were found to be higher in GDM as compared to non-GDM ($p = 0.774$, $p = 0.201$, and $p = 0.267$, respectively). The sTfR/ferritin ratio was lower in the GDM as compared to the healthy controls ($p = 0.326$). Furthermore, the correlation between sTfR/ferritin ratio and insulin resistance was slightly negative ($r = -0.301$, $p = 0.347$). These results suggest that sTfR may be used for the estimation of accurate iron status in the body during gestational diabetes. However, longitudinal studies are required to propose the utility and a cutoff value for this biomarker.

Keywords sTfR · Ferritin · sTfR/ferritin index · GDM

Introduction

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy [1]. It is a common metabolic abnormality with a prevalence ranging from 1 to 28% globally [2] and 17% in Pakistan [3]. Moreover, women who acquire GDM are at a 60% likelihood of acquiring type 2 diabetes mellitus (T2DM) later in life compared to their normal counterparts [4].

GDM has a multifactorial pathophysiology, with obesity, a positive family history of T2DM and hypertension being some of the more common causes [5]. Its incidence is increasing with the rising trends in obesity, sedentary life style, and older age [6] .

Inflammation appears to have an important role in triggering insulin resistance [7, 8], triggering elevation of acute phase proteins like haptoglobin, CRP, serum amyloid a, transferrin, and ferritin [9]. Recent findings indicate a significant correlation between increased iron levels and the pathogenesis of GDM and T2DM [10]. Numerous mechanisms have been proposed for the role of iron in the pathogenesis of diabetes. Iron overload can result in the formation of free radicals through the Fenton reaction which can affect the differentiation of beta cells of the islets of Langerhans of the pancreas, thereby affecting insulin production. Secondly, free radicals can result in an increased transcription of the pro-apoptotic genes which can result in the overstimulation of the apoptotic machinery of the beta cells. A mechanism of cell death dependent on excessive

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cellular iron levels called ferroptosis is also implicated in the pathogenesis of GDM [11].

Biomarkers of iron status such as serum ferritin and serum transferrin receptor (sTfR) protein have been used to investigate the association between iron status and the risk of GDM. Most studies in the medical literature indicate a positive association between ferritin levels and GDM [12–15] with the risk of this association increasing in the second trimester of pregnancy [14].

Ferritin provides a good estimate of the iron stores of the body, but being an acute phase reactant protein, its levels significantly rise with systemic inflammation, which is also one of the defining features of the insulin resistance observed in GDM [16]. A longitudinal prospective cohort study carried out on multiracial pregnant women in 2016 took into account the effect of numerous confounding factors, such as the inflammatory status during pregnancy by controlling for C-reactive protein (CRP) levels and arrived at the conclusion that ferritin levels were significantly raised as early as 10–14 weeks of gestation [14]. Furthermore, sTfR is also a useful indicator of iron depletion [17].

Transferrin and iron form a complex which binds to the transferrin receptors found on the cell surface which internalizes iron into the cells. These transferrin receptors are then cleaved from the surface of the cells and enter the bloodstream to form sTfR. In states of iron depletion, the cells respond by increasing the production of transferrin receptors; subsequently, as more of these receptors are produced, more are cleaved from the cell surfaces, increasing the levels of sTfR in blood [18].

This indicates the need to carry out more studies to elucidate the exact nature of the association between sTfR levels and the risk of GDM. Through this research, we aim to determine the relationship between the two markers of iron and the GDM. It will also help establish which marker, if any, is more suitable for estimation of iron levels and recognition of diabetic pregnant females.

Material and methods

This study was conducted between 2014 and 2015. A total of 88 pregnant females were recruited for this case-control study in the third trimester (28–36 weeks) at the antenatal care clinics of Aga Khan University and Abbasi Shaheed Hospital in Karachi, Pakistan. The cohort was divided into two groups: group A (n = 44) healthy normoglycemic pregnant women (controls) and group B (n = 44) GDM (case) according to the criteria proposed by the IADPSG, i.e., a fasting glucose ≥92 mg/dL and/or 1 h: ≥180 mg/dL (10.0 mmol/L) and/or 2 h: ≥153 mg/dL (8.5 mmol/L) (when any of the following plasma glucose values are exceeded, the subject is considered as having GDM) [19].

Exclusion criteria for this study included: women with diabetes, hypertension, thyroid diseases, assisted or high-risk pregnancy, any pregnancy-related disease (like preeclampsia, cervical insufficiency, vaginal bleeding, etc.), any acute or chronic systemic infections, or any other condition affecting the feto-maternal well-being.

Body mass index (BMI) was calculated by using the formula: weight in kg/height in m^2 [20] by measuring weight in kilogram on a digital weighing scale and standing body height in inches (converted into meters) by the height scale (floor type ZT-120 EVERICH, China).

Under aseptic measures, about 10 mL of venous blood was drawn from each participant, 6 mL in serum separating tubes (SST) (BD Diagnostics) for serum extraction by centrifugation and 4 mL in EDTA tubes containing tubes (BD Diagnostics) for hemoglobin analysis. The serum sample obtained was immediately frozen at −80 °C till further analysis.

Hemoglobin (gm/dL) was measured on Sysmex (21 Cat No. KX21 manufactured by Kobe, Japan). Serum iron was analyzed by enzymatic colorimetric method (Cat # 61075 Bio Merieux S.A., France) (reference range: male 0.65–1.70 mg/dL; female 0.5–1.70 mg/dL). Serum ferritin and soluble transferrin receptor levels in serum samples were determined using a commercially available sandwich ELISA kit (Cat # BC- 1025; BioCheck, USA; Cat # YHB2785Hu, YH Bioresearch China, respectively). The least detectable concentration of human sTfR was 0.5 ng/mL. The sTfR to ferritin ratio was calculated considering <1 as high risk and >1 as low risk [21].

Statistical analysis

Statistical analysis of the data was achieved using the SPSS 19 for Windows software package (SPSS Inc., Chicago, IL). A descriptive statistical analysis of continuous variables was performed. Data on continuous variables, i.e., biophysical and biochemical parameters were presented as mean + standard deviation (SD). Statistical comparisons were performed by using Mann Whitney U test for non-parametric variables. Associations between circulating sTfR levels and BMI were determined using Spearman's rank correlation. Associations were considered significant if $p < 0.05$.

Results

The overall cohort of 88 pregnant females was divided into 2 groups using IADPSG criteria as GDM and non-GDM. The results of the biophysical and biochemical parameters of this study are shown in Table 1. With the mean ± SD = 26.35 ± 0.71 years, there was no significant difference between the groups. Similarly there was no significant difference in their weight (mean ± SD = 62.05 ± 1.75 kg)

Table 1 Biophysical and biochemical parameters of the study subjects

Variables	Group A (control) (non- GDM) (Mean ± SD) (n = 44)	Group B (cases) (GDM) (Mean ± SD) (n = 44)	p value
Age (years)	25.44 ± 0.692	27.27 ± 0.772	0.53
Weight (kg)	59.2 ± 1.73	64.90 ± 1.78	0.32
BMI (kg/m^2)	23.8 ± 0.69	25.80 ± 0.655	0.069
Fasting blood glucose (FBG) mmol/L	4.884 ± 0.16	5.85 ± 0.266	0.001
Insulin (mIU/L)	39.61 ± 1.34	13.01 ± 0.40	< 0.001
HOMA-IR	4.84 ± 0.65	6.74 ± 0.577	<0.001
hsCRP (mg/L)	3.45 ± 1.69	5.89 ± 1.35	0.267

and BMI (mean ± SD = 24.8 ± 0.67 kg/m2). The insulin resistance (HOMA-IR) was significantly high in GDM as compared to non-GDM subjects ($p < 0.001$), however, the insulin levels were found to be significantly low in the GDM group as compared to the non GDM group. Results of the iron status of the study subjects are shown in Table 2. Hemoglobin was lower than the reference range in the total cohort with a mean ± SD = 10.70 ± 0.175; however, it was considerably lower in GDM females. There was a non-significant decrease noted in iron levels of GDM subjects as compared to non-GDM subjects ($p = 0.758$). Serum levels of sTfR, ferritin, and hs-CRP were found to be non-significantly higher in GDM as compared to non-GDM ($p = 0.774$, $p = 0.201$, and $p = 0.267$, respectively). The sTfR/ferritin ratio was lower in the GDM as compared to the healthy controls ($p = 0.326$). The correlation between sTfR/ferritin ratio and insulin resistance (HOMA-IR) was slightly negative, yet we failed to observe any significant difference among the groups ($r = -0.301$, $p = 0.347$) Fig. 1.

Discussion

Ferritin is the major iron storage protein [22], and its concentrations are considered to be a good proxy for body iron stores.

Table 2 Iron status of the study subjects

Variables	Group A (control) (non- GDM) (Mean ± SD) (n = 44)	Group B (cases) (GDM) (Mean ± SD) (n = 44)	p value
Hemoglobin (g/dL)	10.84 ± 0.17	10.56 ± 0.18	0.212
Serum iron (μg/dL)	100.11 ± 10.45	89.15 ± 13.33	0.758
sTfR (ng/mL)	16.74 ± 0.725	19.16 ± 3.07	0.774
Ferritin(ng/mL)	16.29 ± 11.12	22.38 ± 8.74	0.201
sTfR/ferritin ratio	1.02	0.85	0.326

But it being an acute-phase reactant, ferritin levels can also increase in conditions with subclinical systemic inflammation, which is associated with insulin resistance in GDM [23]. There is an increasing body of evidence that suggests that higher iron stores may be associated with an increased risk of types 1 and 2 diabetes [24], including our previous reports that showed a similar trend in obese and diabetic individuals [25, 26]. The link postulated is of a systemic inflammation in GDM, as indicated by higher levels of serum CRP and/or other inflammatory cytokines [27, 28], but it is not clear if this increase reflected inflammation is due to excess iron stores.

In this study we report, no difference in the ferritin levels, sTfR: ferritin ratio and hs-CRP levels in females with and without gestational diabetes. Among iron biomarkers, ferritin has been most often investigated in relation to diabetes. Our findings are consistent with other studies, conducted on women in Turkey and the UK, which showed a lack of association with higher-than-normal ferritin levels and the risk of GDM [29, 30]. However, in a prospective study, higher ferritin concentrations and a lower sTfR:ferritin ratio were associated with an elevated risk of GDM [31]. This difference may be explained by the different timings of blood sampling as well as the cross-sectional nature of our study.

Inflammatory cytokines have been shown to induce ferritin synthesis in experimental models [32], and sTfR is assumed to reliably reflect the degree of tissue iron supply. sTfR, a marker of tissue iron insufficiency, has also been understudied in the context of GDM risk but we were unable to find any association with GDM in the present study. Similar to our finding, no significant association was observed between sTfR levels and GDM risk in studies conducted recently [33, 34, 35]. Our study is unique in the sense that we further examined the sTfR/ferritin ratio, a measure that captures both cellular iron demand and the availability of body iron stores. The added value of using sTfR:ferritin ratio, as opposed to isolated measurements of sTfR and ferritin, is that it covers the full spectrum of iron homeostasis, from normal, healthy iron stores to mild or substantial functional iron deficiency [35]. The sTfR:ferritin ratio offers greater sensitivity and

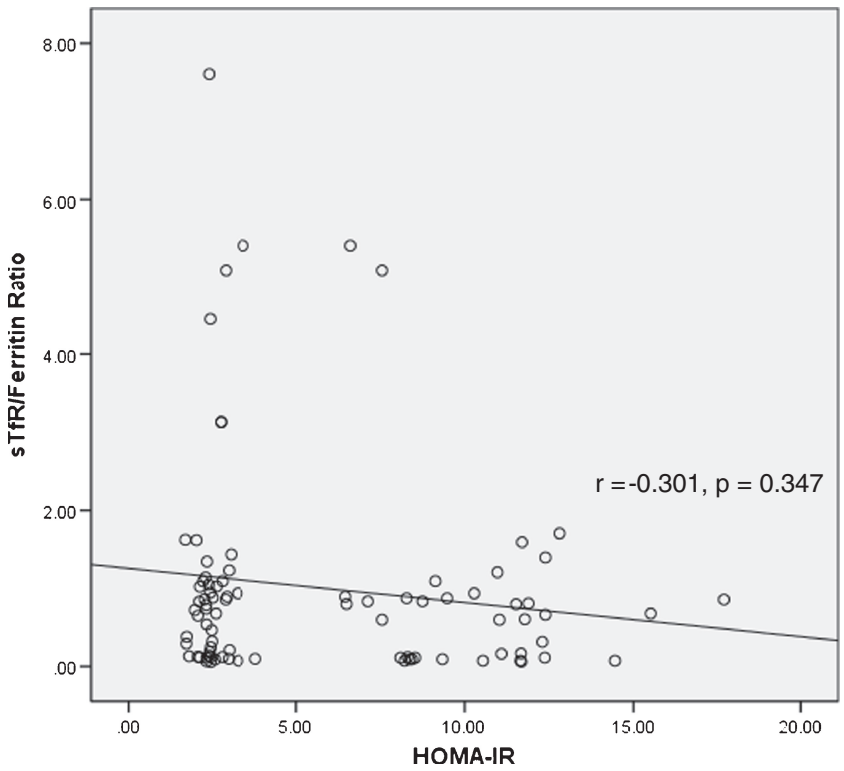

Fig. 1 Correlation of sTfR/ ferritin ratio and HOMA-IR

specificity in characterizing iron status than individual measurements of ferritin and sTfR and is considered to be particularly useful when individual measurements yield ambiguous results [23]. We report that a slightly negative relation of sTfR:ferritin ratio with GDM, even though we found no difference in their individual values between groups.

Our findings are plausible; since the females were diagnosed by a stricter criterion followed by early introduction of treatment which might have decreased the harmful effects of ferritin as a pro-oxidant, by inhibiting the generation of reactive oxygen species, oxidative stress and minimizing beta cell damage or insulin signaling. The limitations of this study are that we collected samples at the time of glucose tolerance test, i.e., between 24 and 28 weeks of gestation, and were unable to record/measure the effect of dietary iron intake on these biomarkers. Our study, however, examined the conventional and novel biomarkers of iron status to account for the inflammatory state in our subjects, which is its strength.

Conclusion

These results suggest that sTfR may be used for the estimation of true iron status in the body during gestational diabetes. However, longitudinal studies are required to propose the utility and a cutoff value for this biomarker. The findings from this pilot study can be used as a stepping stone to design further longitudinal and prospective studies among multiracial pregnant women to assess the role of maternal iron stores in the development of GDM from as early as the first trimester and follow till birth and to assess the cord blood levels.

Acknowledgements The authors would like to thank the Department of Biological and Biomedical Sciences, Aga Khan University for funding this project. The authors would also like to acknowledge the patients who were enrolled in this project along with the technical staff of the project.

Author contribution SSF and FA were involved in conception and design, analysis, and interpretation of data. HS, SK, and SA were involved in acquisition of data, analysis, and interpretation of data. All authors took active part in drafting the article and revising it critically for important intellectual content. All authors approved the final version submitted for publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Funding source Department of Biological and Biomedical Sciences, Research module fund

Ethical approval An ERC approval (2832-BBS-ERC-13) was obtained and every participant signed an informed written consent.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):47–54
DOI 10.1007/s13410-016-0541-2

ORIGINAL ARTICLE

Insulin therapy in women with pregestational type 2 diabetes and its relevance to maternal and neonatal complications

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Received: 26 August 2016 / Accepted: 8 December 2016 / Published online: 19 December 2016

Abstract The objective of this study is to assess the insulin requirement, its determinants, and its association with maternal complications and neonatal outcome in women with pregestational type 2 diabetes mellitus. One hundred two insulin treated pregnant women with pre-existing type 2 diabetes and those clinically diagnosed with type2 DM during pregnancy were selected. Insulin dose, distribution, relation with meal was assessed. Statistical analysis was done and insulin requirement was correlated with maternal factors and fetal outcome. Insulin dose at the 1st trimester was 32.65 ± 23.11 units/day, i.e., 0.52 U/kg/day of pre-pregnancy weight, which significantly increased to 47.62 ± 29.54 U/day at delivery i.e., 0.76 U/kg/day ($p < 0.0001$). Insulin dose was positively correlated to fasting and 2 h postprandial plasma glucose at diagnosis. Pre-dinner insulin requirement was significantly higher than pre-breakfast in the 3rd trimester (*P* value: 0.018). 19.6% neonates had a low birth weight, 5.8% had macrosomia, and 18.63% had neonatal hypoglycemia. Subjects on insulin analog showed a lower risk of low birth weight (17.4%) and macrosomia (Nil) versus those on conventional insulin with 21.6 and 8.1%, respectively. Insulin requirement in type 2 diabetes pregnancies progressively increases from the 1st trimester till delivery. Meal-related assessment needs attention in Indian population due to their varied dietary culture. Low birth weight is more frequent than macrosomia in our population. More studies are needed to ascertain the concept of a better neonatal outcome with insulin analog.

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Keywords Pregestational diabetes mellitus · Insulin · Fetal outcome

Recent evidences show that over 415 million people worldwide have diabetes, which is expected to become 652 million by 2040. 78.3 million people with diabetes live in South East Asia. India has 68.1 million people with diabetes (appx. 8.8%), and half of them are undiagnosed. Globally, 199.5 million of women have diabetes, which is expected to increase to 313.3 million in 2035 [1]. Hyperglycemia is one of the most common medical conditions women encounter during pregnancy. The International Diabetes Federation (IDF) data says that one in six live births (16.8%) born of women with some form of hyperglycemia in pregnancy (20–49 years). FIGO (International Federation of Gynecology and Obstetrics) in their recently published guidelines [2] classified hyperglycemia in pregnancy into two categories: (a) gestational diabetes mellitus (GDM) and (b) diabetes in pregnancy (DIP) which is further subclassified as either pre-existing type 2 DM or type 1 diabetes with pregnancy or the hyperglycemia first detected at any time during the course of pregnancy, if meets the criteria for diagnosis of diabetes in the nonpregnant state. The stated criteria are fasting plasma glucose (FPG) ≥7.0 mmol/L or 126 mg/dL, and/or 2-h 75-g oral glucose tolerance test (OGTT) value ≥11.1 mmol/L or 200 mg/dL, or random plasma glucose (RPG) ≥11.1 mmol/L or 200 mg/dL associated with signs and symptoms of diabetes. In DIP, the susceptibility to complications is more because of the higher degree of hyperglycemia and the uncertainty as to whether the onset of hyperglycemia was prior to pregnancy or developed during early pregnancy. Though, diabetes detected for the first time in pregnancy might be type 1 or type 2

DM, a diagnosis of type 2DM is more likely. Compared with GDM, DIP is more likely to be detected as early as the 1st trimester (TM), provided appropriate testing is commenced. Although, 16% of hyperglycemia in pregnancy might be DIP, the majority (84%) is due to GDM. Furthermore, globally and more so in Asia, the age of onset of diabetes and pre-diabetes is declining, while the age of childbearing is increasing. Additionally, there is a rising prevalence of overweight and obese women of reproductive age; thus, women entering pregnancy have higher risk factors that make them susceptible to hyperglycemia during pregnancy [2]. Many recommendations define women with any type of diabetes, when attains pregnancy as pregestational diabetes mellitus (PGDM) [3] and those diagnosed to have hyperglycemia during pregnancy, though GDM, but is clinically pre-GDM or overt pre-GDM.

Pregnancy is a diabetogenic state that exacerbates pre-existing diabetes. Due to various counter-regulatory hormones, metabolism changes noticeably during pregnancy. Both basal and postprandial glucose metabolism gradually change during the course of pregnancy to meet the maternal and fetal nutritional demands. Optimal glycemic control is pivotal to the successful outcome of diabetes in pregnancy. It is imperative to achieve euglycemia during pregnancy with the goal of fasting blood glucose <90 mg, 2 h post meal as <120 mg% and HbA1c < 6%. While targeting these goals, we should also take care to avoid severe hypoglycemia. These goals are best obtained with diet, exercise, and insulin treatment, often a multiple-dose insulin regimen or insulin pump is required. Insulin therapy is the mainstay to achieve euglycemia during pregnancy complicated by any form of diabetes. Euglycemia for pregnancy can be best achieved through basal-bolus regimen of insulin therapy with judicious use of home monitoring of blood glucose (HMBG). Importantly, innovations in home blood glucose monitoring and insulin administration devices have provided the technology needed to not only allow women to successfully survive pregnancy but also to decrease the risks of diabetic fetopathy to those of the nondiabetic population [3, 4]. There is scanty data available on dose, frequency, and meal-related distribution of insulin therapy in type 2 women with diabetes during pregnancy.

In this retrospective noncontrolled observational study, we have tried to evaluate these parameters and their association with the maternal characteristics at diagnosis of pregnancy. Also, the change in the insulin requirement from the 1st to 3rd trimester and its effect on maternal and fetal complications was studied. This has been shown schematically in Fig. 1. Step I shows the assessment of baseline maternal characteristics at the diagnosis of pregnancy. These characteristics were correlated with the total insulin dose/day at the 1st trimester. Further, the dose requirement at the 1st trimester was compared with that of the 3rd trimester (step II) considering maternal and fetal complications as outcomes.

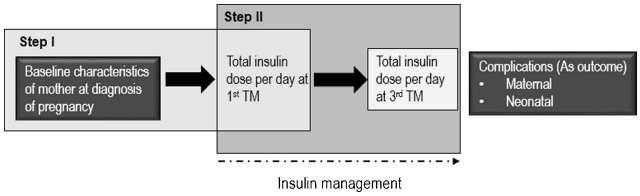


Fig. 1 Conceptual flow of the analysis

Materials and methods

A total of 120 pregnant women with DIP, as defined by FIGO [2], i.e., either pre-existing type 2 diabetes or those diagnosed to have diabetes for the first time during pregnancy (overt pre-GDM), attending a tertiary care diabetes center in Central India, during the period 2008 to 2015 were included in the study. Sample selection (through convenient sample method) has been depicted through Fig. 2. Out of these, 15 patients have opted to terminate pregnancy due to a high glycosylated hemoglobin A1c or other associated complications. There was one case with intrauterine death (IUD), one patient had an extremely high BMI, and one was on a diet therapy throughout the gestational period. This resulted into 102 cases for final evaluation. The study was approved by the institutional ethics committee, informed consent was obtained for using the data from the case file, and their privacy rights were maintained. Detailed history with pre-pregnancy weight and present 3 days dietary recall was taken for each case. Anthropometry measurements were taken. Biochemistry was done by fully automated Cobas c111 analyzer developed by Roche, glycosylated hemoglobin A1c was done by HPLC (high performance liquid chromatography) method with Bio-Rad D-10

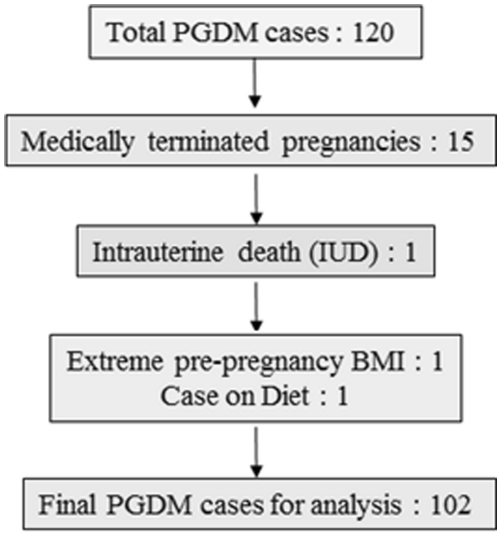


Fig. 2 Sample selection for the study

analyzer, thyroid function test was performed by electrochemiluminescence immunoassay (ECLIA), micro-angiopathy, and macro-angiopathy assessment were done through clinical examination and with resting ECG, micro-albuminuria was done by immunoturbidimetric assay and fundus examination through Zeiss direct system.

Subjects were initiated primarily on basal-bolus insulin regimen to achieve the goal of fasting blood glucose as less than 90 mg% and 2 h postprandial as less than 120 mg%. Few of them continued with premixed insulin. Their fasting, post-breakfast, pre-lunch, post-lunch, pre-dinner, post-dinner, and 3 am blood glucose were monitored regularly. Counseling for HMBG and self-adjustment of minor insulin doses was done. After achieving the glycemic targets, the insulin requirement was assessed from the diagnosis of pregnancy till delivery. Insulin dose requirement was calculated with respect to patient age, age of onset of diabetes, duration of diabetes, pre-pregnancy weight, pre-pregnancy BMI, fasting plasma glucose, postprandial plasma glucose and HbA1c at diagnosis of pregnancy, need of intermediate acting insulin, weight gain during pregnancy, and relation with meals, i.e., breakfast, lunch, and dinner. Eventually, maternal and fetal complications were assessed.

Statistical methods

The descriptive statistics like mean and standard deviation were obtained for continuous variables, while frequencies were obtained for variables defined on nominal scale. Relationship of different maternal characteristics with total insulin requirement at the 1st trimester was obtained using *Pearson's correlation coefficient* for continuous characteristics, and *t test for independent samples* for categorical characteristics. All the analysis was performed using SPSS 18.0 (SPSS Inc.) software, and statistical significance level was set at 5%.

Results

The sample selection in the study has been shown pictorially in Fig. 2. There were a total of 120 patients of DIP during the observation period; however, the analyses were performed on 102 eligible cases. Among these, 45 (44%) had known diabetes, while 57 (56%) cases were overt pre-GDM. Out of 45 known diabetics, 43 were type 2 DM cases, while 2 were known IGT (impaired glucose tolerance). The descriptive statistics for different patient characteristics are given in Table 1. The mean age of onset of DM in patients with known pre-existing T2DM was 29.00 ± 4.79 years, while the overall mean age of patients was 30.17 ± 4.25 years. There were 21 (20%) cases with a past history of GDM, 51 (50%) cases with bad obstetric history, and 75 (73.53%) cases with a family

Table 1 Descriptive statistics for different patient characteristics (n = 102)

Characteristics	Descriptive statistics
Age at onset of diabetes (yrs.) [M ± SD]†	29.00 ± 4.79
Age at gestation (yrs.) [M ± SD]	30.17 ± 4.25
Duration of DM before gestation (yrs.) [no. (%)] (n = 43)†	
≤1	19 (44.18)
>1	24 (55.81)
Past history of GDM (yes) [no. (%)]	21 (20)
Obstetric history—Gravida [no. (%)]	
1	29 (28.43)
>1	73 (71.57)
BOH (yes) [no. (%)]	51 (50)
Family history of diabetes (yes) [no. (%)]	75 (73.53)
Pre-pregnancy weight (Kg) [M ± SD]	62.59 ± 13.62
Pre-pregnancy BMI (Kg/m^2) [M ± SD]	26.32 ± 5.02
HbA_1c% at diagnosis of pregnancy [M ± SD]	7.39 ± 1.40
Fasting blood sugar (mg/dl) [M ± SD]	121.50 ± 44.03
Post-meal blood sugar (mg/dl) [M ± SD]	196.50 ± 71.18
Hypothyroidism (yes) [no. (%)]	22 (21.57)
Total insulin—1st trimester (units/day) [M ± SD]	32.65 ± 23.11
Before breakfast	9.15 ± 7.02
Before lunch	9.63 ± 7.38
Before dinner	10.02 ± 6.93
Bed time	5.31 ± 4.73
Total insulin—3rd trimester (units/day) [M ± SD]	47.62 ± 29.54
Before breakfast	12.74 ± 9.28
Before lunch	15.10 ± 9.73
Before dinner	15.89 ± 9.85
Bed time	8.00 ± 6.90
Total calorie intake (cal.)	
At breakfast	270.45 ± 127.95
At lunch	427.11 ± 123.08
At supper	463.86 ± 118.59
Weight gain in pregnancy (Kg) [M ± SD]	8.08 ± 3.53
Gender (baby): male [no. (%)]	53 (51.96)
Baby birth weight (Kg) [M ± SD]	2.76 ± 0.54
Neonatal complications [no. (%)]	
Requiring PBU admission	12 (11.76)
Macrosomia (>3.5 kg)	6 (5.88)
Low birth weight (<2.5 kg)	20 (19.61)
Neonatal hypoglycemia	19 (18.63)
Neonatal hyperbilirubinemia	4 (3.92)
Neosepsis	1 (0.98)
Maternal complication [no. (%)]	
PIH	14 (13.73)

†Only cases with known type 2 DM before pregnancy

history of diabetes. The mean pre-pregnancy weight of patients was 62.59 ± 13.62 kg, and pre-pregnancy BMI was 26.32 ± 5.02 kg/m^2. Other baseline features at diagnosis like HbA_1c was 7.39 ± 1.40, fasting plasma glucose (FPG) was 121.50 ± 44.03 mg/dl, and postprandial plasma glucose (PMPG) was 196.50 ± 71.18 mg/dl. Of them, 22 (21.57%) subjects had hypothyroidism. Pregnancy-induced hypertension (PIH) was observed in 14 (13.73%) mothers. The total per day insulin requirement at the 1st trimester was 32.65 ± 23.11 units, while per kg pre-pregnancy body weight requirement was 0.522 units/kg. The requirement increased to 47.62 ± 29.54 mg/dl at the time of delivery, and per kg pre-pregnancy body weight requirement was 0.76 units/kg. The day wise insulin requirement revealed that it was the highest at dinnertime both at the 1st and 3rd trimester as compared to morning and afternoon. The average weight gain in pregnancy was 8.08 ± 3.53 kg, and the average baby birth weight was 2.76 ± 0.54 kg. As regards neonatal complications, 20 (19.61%) babies had a low birth weight, while 19 (18.63%) had a neonatal hypoglycemia. There were 6 (5.8%) babies with macrosomia. There was no case of congenital malformation.

Factors and their relatedness with insulin dose (step I)

As per the conceptual flow (Fig. 1), the statistical relevance of baseline features with total insulin dose required during the 1st trimester was established, with the results are shown in Table 2. Age at onset of DM in patient subgroup with known T2DM was insignificantly correlated with the total insulin dose at the 1st trimester with a coefficient of −0.243 (*P* value: 0.124). In the same subgroup, the duration of DM showed insignificant difference of mean total insulin requirement per day as indicated by a *P* value of 0.282. The mean dose required for patients with the past history of GDM (30.95 ± 29.52 units/day) was insignificantly different from those without history (33.10 ± 21.32 units/day) as indicated by a *P* value of 0.757. Patients with and without bad obstetric history also did not show statistically significant difference in the mean dose requirements (*P* value: 0.922). The dose requirement was uninfluenced by the family history of diabetes, pre-pregnancy BMI, and HbA_1c at the diagnosis of pregnancy in patients as revealed by *P* values 0.147, 0.977, and 0.129, respectively. However, fasting blood sugar at diagnosis showed a significant positive correlation (0.219) with insulin dose requirement in the 1st trimester with an associated *P* value of 0.03. At the 3rd TM, the correlation was slight negative (−0.013) and statistically insignificant (*P* value: 0.898) with insulin dose. Moreover, post-meal blood sugar was significantly correlated (0.179) with dose requirement (*P* value: 0.073), which was significant at 10% level of confidence; while at the 3rd TM, the correlation was 0.019 (*P* value: 0.852).

Change in the insulin dose requirement (step II)

The insulin requirement of the 1st trimester was 32.65 ± 32.11/day while the total insulin dose requirement at the 3rd trimester showed a mean of 47.62 ± 29.54 U/day (Table 1). This increase in the mean dose requirement from the 1st to the 3rd trimester was statistically highly significant with $P < 0.0001$. The overall requirement at the 3rd trimester was nearly 1.46 times more than that of the 1st trimester.

Moreover, the split of insulin requirement at different times during the day at the 1st trimester revealed a linear increase in dose with the day progression. The insulin requirement was higher at supper (10.02 ± 6.93 U/day) than at breakfast (9.15 ± 7.02 U/day). The mean change in the dose requirement between breakfast and supper was 0.87 units, which was statistically insignificant (*P* value: 0.408). At the 3rd trimester, the mean insulin requirement at supper (15.89 ± 9.85 U/day) was higher than at breakfast (12.74 ± 9.28 U/day). The mean difference of doses between these time points was 3.15 units, which was statistically significant with a *P* value of 0.018. Further, the mean change in the insulin requirement at breakfast between the 1st and 3rd trimester (3.59 U) was significant with a *P* value of 0.0042, while at supper, the mean change (5.87 U) was highly significant with $P < 0.0001$. The change in bedtime insulin NPH/detemir from diagnosis of pregnancy till delivery (2.97 U) was significant with a *P* value of 0.0005.

The increase in the insulin dose requirement from breakfast to dinner at diagnosis of pregnancy as well as at delivery corroborates with a high total calorie intake of the population at supper (463.86 ± 118.59 cal.) as against breakfast (270.45 ± 127.95 cal.).

Insulin dose requirement and its relation with maternal and neonatal complications as outcome

Maternal complications like hypothyroidism and pregnancy-induced hypertension (PIH) were observed in the study sample. Table 3 reveals that in patients with hypothyroidism, the mean total per day dose requirement at the 1st trimester was 30.59 ± 18.57 units; while at delivery, it was 54.23 ± 39.96 units. The change in the dose was statistically significant (*P* value: 0.017). Also, in patients without hypothyroidism, the change in the insulin dose was significant with $P = 0.002$. Overall, the mean increase in hypothyroidism group (~24 units) was higher as compared to that of nonhypothyroidism group (~13 units). However, this difference was statistically insignificant with a *P* value of 0.1616. Further, in mothers who developed PIH, the mean total per day insulin requirement at the 1st trimester was 22.92 ± 9.46 units, while at the time of delivery, it was 43.86 ± 30.03 units. The increase was statistically significant

Table 2 Relationship of different maternal characteristics with total insulin requirement per day during the 1st and the 3rd trimester (n = 102)

Baseline maternal characteristics	Insulin dose—1st TM		Insulin dose—3rd TM	
	Descriptive statistics	*P* value	Descriptive statistics	*P* value
Age at onset of diabetes (yrs.) [r]	−0.243	0.124†	−0.251	0.099†
Overt pre-GDM	29.95 ± 22.93	0.174*	40.75 ± 27.61	0.006*
Pre-GDM (T2DM)	36.30 ± 23.11		57.47 ± 30.36	
Duration of DM before gestation (yrs.) [M ± SD]				
(Among pre-GDM T2DM)				
≤1	31.23 ± 24.90	0.282*	46.89 ± 30.04	0.601*
>1	36.03 ± 18.13		50.21 ± 29.41	
Past history of GDM [M ± SD]				
Yes	30.95 ± 29.52	0.757*	51.94 ± 30.43	0.547*
No	33.10 ± 21.32		47.12 ± 29.68	
Obstetric history—Gravida [M ± SD]				
1	30.90 ± 19.77	0.764*	43.60 ± 25.57	0.296*
> 1	32.29 ± 23.42		49.91 ± 31.34	
Bad obstetric history [M ± SD]				
Yes	32.32 ± 19.52	0.922*	52.56 ± 32.88	0.142*
No	32.78 ± 24.47		43.70 ± 26.01	
Family history of DM [M ± SD]				
Yes	34.39 ± 24.72	0.147*	50.11 ± 29.08	0.258*
No	27.89 ± 17.51		42.07 ± 31.27	
Pre-pregnancy BMI [r]	0.002	0.977	0.067	0.509
HbA_1c at diagnosis of pregnancy [r]	0.151	0.129	0.016	0.868
Fasting blood sugar [r]	0.219	0.030	−0.013	0.898
Post-meal blood sugar [r]	0.179	0.073	0.019	0.852

r Pearson's correlation coefficient, *M* mean, *SD* standard deviation

†Only cases with known type 2 DM before pregnancy,

*Obtained using *t-test for independent samples*

with a *P* value of 0.025. In patients without PIH, the difference in the total daily dose requirement was also significant (*P* value: 0.001). Overall, the mean increase in PIH group (~21 units) was higher than that of non-PIH group (~14 units). However, this difference was statistically insignificant with a *P* value of 0.249. The occurrence of maternal complications like hypothyroidism and PIH in overt PGDM and type 2 DM groups was insignificantly different (Table 4). There was no case of maternal hypoglycemia reported.

As regards neonatal complications, 20 (19.61%) babies had a low birth weight (birth weight less than 2.5 kg), 6 (5.8%) had macrosomia (birth weight more than 3.5 kg), and 19 (18.63%) had neonatal hypoglycemia. Among the low birth weight cases, 1 (5%) had hypoglycemia, 5 (25%) required PBU (premature baby unit) admission, and the rest had no complications. In the normal birth weight category, 17 (22.4%) had hypoglycemia and 6 (7.9%) cases required PBU. Among babies with macrosomia, 1 (16.7%) had hypoglycemia, and 1 (16.7%) case required PBU admission. The proportion of neonatal cases with complications like macrosomia, low birth weight, neonatal hypoglycemia was insignificantly different between pre-existing type 2 DM and overt pre-GDM groups (Table 4).

The insulin requirement in cases with neonatal complications was also studied (Table 3). In low birth weight, the change in the requirement of insulin from the 1st to 3rd trimester (at delivery) was statistically insignificant, as indicated by a *P* value of 0.246. Similarly, in macrosomia cases, the change was statistically insignificant with a *P* value of 0.702. However, in normal babies, the change in the requirement was highly significant with a *P* value of 0.0001. In babies with hypoglycemia, the insulin requirement in the 3rd TM was significantly higher than that of the 1st TM (P = 0.047). Similar was the observation in babies without neonatal hypoglycemia (*P* value: 0.001).

The effect of the type of insulin was also studied in patients through Sankey chart (Fig. 3). There were 74 mothers with conventional insulin (Regular, Regular + NPH, premixed-50/50) treatment throughout gestation period, while 25 mothers were on insulin analogs (rapid acting insulin analog insulin

Table 3 Relevance of maternal and infant complications with insulin requirement (n = 102)

Complications	Total per day insulin requirement at		P value*
	1st TM	3rd TM	
Maternal complications			
Hypothyroidism			
Yes (n = 22)	30.59 ± 18.57	54.23 ± 39.96	0.017
No (n = 80)	33.23 ± 24.30	45.80 ± 26.00	0.002
PIH			
Yes (n = 14)	22.92 ± 9.46	43.86 ± 30.03	0.025
No (n = 88)	34.09 ± 24.19	48.22 ± 29.59	0.001
Neonatal complications			
Body weight			
Low birth weight (n = 20)	31.20 ± 19.04	38.85 ± 21.88	0.246
Normal (n = 76)	31.72 ± 22.77	49.17 ± 30.72	0.0001
Macrosomia (n = 6)	49.17 ± 35.80	57.17 ± 34.61	0.702
Hypoglycemia			
Yes (n = 19)	26.37 ± 19.16	44.47 ± 32.92	0.047
No (n = 83)	34.11 ± 23.80	48.34 ± 28.88	0.001

*Obtained using *paired t test*;

**Obtained using *t test for independent samples*;

‡ Obtained using *one-way ANOVA*

aspart and long acting insulin analog, insulin detemir) during the period. In subjects on conventional insulin group, 16 (21.62%) babies had a low birth weight (birth weight less than 2.5 kg.), followed by 14 (18.92%) with neonatal hypoglycemia (blood glucose less than 40 mg%), 9 (12.16%) required PBU (premature baby unit) admission, and 6 (8.11%) had macrosomia (birth weight more than 3.5 kg). In the insulin analog treated group, 4 (17.39%) had a low birth weight, 5 (21.74%) had neonatal hypoglycemia, while 3 (12%) babies required PBU, and there was no case of macrosomia. There were 2 cases with the change of treatment from conventional to analog, while 1 case with the change from analog to conventional, and thus they were not included in the analysis.

Discussion

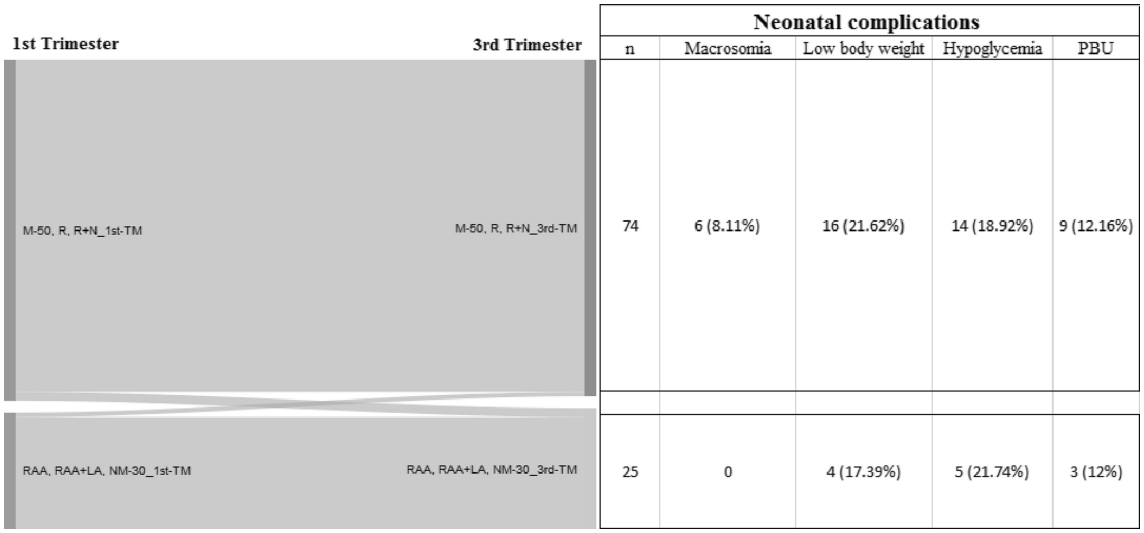
Subcutaneous insulin administration is the mainstay of intensified therapy for diabetes in pregnancy (DIP), i.e., pre-existing diabetes in pregnancy. Basal-prandial insulin delivery through a multiple-injection regimen or CSII is most effective [5]. Type 2 diabetes women on insulin therapy may require an initial total daily dose of 0.7–1.0 units/kg actual body weight, adjusted according to subsequent blood glucose concentrations. Obese women may require a higher insulin dosage, and insulin requirements may double or triple during the course of pregnancy [6]. In our study, all subjects were primarily treated with basal-bolus

Table 4 Complications according to type 2 DM and clinically PGDM mothers

Complications	Type 2 DM (n = 42)	Clinically PGDM (n = 60)	P value*
Maternal complication			
Hypothyroidism	10 (23.8%)	12 (20.0%)	0.8291 (NS)
PIH	9 (21.4%)	5 (8.30%)	0.1097 (NS)
Neonatal complications			
Requiring PBU admission	3 (7.14%)	9 (15.0%)	0.3682 (NS)
Macrosomia (>3.5 kg)	2 (4.76%)	4 (6.67%)	0.9999 (NS)
Low birth weight (<2.5 kg)	8 (19.5%)	12 (20.0%)	0.9999 (NS)
Neonatal hypoglycemia	8 (19.5%)	11 (18.3%)	0.9999 (NS)
Neonatal hyperbilirubinemia	2 (4.76%)	2 (3.33%)	0.9999 (NS)

NS nonsignificant

*Obtained using *chi-Square test*



	Neonatal complications			
n	Macrosomia	Low body weight	Hypoglycemia	PBU
74	6 (8.11%)	16 (21.62%)	14 (18.92%)	9 (12.16%)
25	0	4 (17.39%)	5 (21.74%)	3 (12%)

Fig. 3 Sankey chart displaying the number of patients according to the type of insulin administered during the 1st and 3rd trimester and the respective neonatal complications

regimen of insulin therapy, and it was observed that to achieve target fasting plasma glucose as less than 90 mg% and 2 h postprandial plasma glucose as less than 120 mg%; they require 0.52 U/kg of pre-pregnancy body weight/day of insulin in the 1st trimester, which gradually increases to 0.76 U/kg of pre-pregnancy body weight/day. The overall requirement at the 3rd trimester was nearly 1.46 times more than that of the 1st trimester. The increment in the dose requirement from the 1st trimester to the 3rd trimester was observed to be less in our population than shown in other studies [7, 8]. The dose requirement of insulin lispro (another rapid acting insulin analog) was observed by Durnwald [9], which is almost similar to our finding. He found 0.58 and 0.98 U/kg of pre-pregnancy body weight insulin lispro needed in the 1st and 3rd trimester, respectively. R Chaudry et al. [10] mentioned that many obese women may require as much as 1.5–2.0 U/kg initially to overcome the combined insulin resistance of obesity and pregnancy. Our data showed that insulin requirement is uninfluenced by pre-pregnancy BMI in these pre-GDM women.

Sapienza AD et al. [11] have shown in their study on gestational diabetes mellitus, a positive correlation between insulin therapy and pre-pregnancy BMI, family history of diabetes, hypertension, prior GDM, prior fetal macrosomia, and HbA1c in univariate analysis. Pre-pregnancy BMI, family history of diabetes, and HbA1c were statistically significant variables in the logistic regression model in this study. Our type 2 diabetes mellitus pre-GDM women did not show any statistically significant relationship of insulin dose requirement with the duration of DM, past history of GDM, bad obstetric history, positive family history of diabetes, pre-pregnancy BMI, and HbA_1c at diagnosis. Insulin requirement did show a statistically significant relation with fasting and postprandial plasma glucose at the diagnosis of pregnancy.

Our study is unique to show the statistically significant increase in insulin doses from pre-breakfast to pre-supper, especially at delivery. The increase in the insulin dose requirement from breakfast to dinner at diagnosis of pregnancy as well as at delivery corroborates with a high total calorie intake of the population at supper as against breakfast. The increase in the mean levels of insulin dose requirement was much higher in women with hypothyroidism and PIH as compared to those without hypothyroidism or without PIH. This might be due to a more insulin resistance in women with hypothyroidism and PIH. The increase in insulin requirement from the 1st trimester to the 3rd trimester was significantly higher in mothers who delivered normal babies (74.5%) as against mothers who delivered low birth weight (19.6%) or macrosomic babies (6%). This indicates that adequate insulin intensification might play an important role for a better fetal outcome, which could be attained through proper glucose monitoring at different times during the day. While comparing the use of conventional human insulin versus insulin analog, there was no significant difference found in neonatal hypoglycemia, low birth weight, or PBU admission. These results are consistent with Pettitt DJ et al. [12] who used insulin aspart and Seshiah et al. [13], Jovanovic et al. [14], and Meccaci et al. [15], when they have used insulin lispro. Deepakala MC et al. [16] have found 12.8% of macrosomia and 11% of babies with a low birth weight in women who were on insulin lispro. Our data has shown 8.11% babies to have macrosomia and all of them were on conventional human insulin. There was no case of macrosomia in women who were on insulin analogs (aspart and detemir). Our limitation was a small sample size of women on insulin analog and thus, further studies are needed to ascertain these observations.

Conclusion

Insulin is the first line therapy recommended for women with diabetes in pregnancy. Though the insulin requirement needs to be individualized, there is scanty data available for the factors affecting the insulin dose requirement, frequency, and relation with various meals during pregnancy. The insulin dose requirement progressively increases from the 1st trimester to the 3rd trimester. Monitoring of all pre and post-meal blood glucose helps to have a better glycemic control. Post-supper blood glucose monitoring needs emphasis. Careful intensification of insulin therapy and the use of insulin analog might help to have better feto-maternal outcomes.

Acknowledgements Dr. Dhananjay Raje for the statistical assistance.

Compliance with ethical standards

Funding None.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval This study is approved by the Institutional Ethics Committee, though it is retrospective noncontrolled observational study. There is no intervention as insulin was given to the women participants as part of the standard of care and not as part of any study; the authors only documented the dose, type, and duration of insulin that were administered to the participants.

Informed consent Informed consent from all the subjects was taken for using their personal data for research purposes.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):55–61
DOI 10.1007/s13410-017-0560-7

ORIGINAL ARTICLE

Type 2 diabetes mellitus and *TCF7L2* gene rs12255372 G/T polymorphism: a meta-analysis involving 7990 subjects

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Received: 8 February 2017 / Accepted: 8 May 2017 / Published online: 7 June 2017

Abstract *The transcription factor 7-like 2 (TCF7L2)* G/T gene polymorphism, also known as rs12255372, has been linked to increases in risk for type 2 diabetes (T2DM), especially in the European population. However, a clear consensus on the subject is still lacking in analyses of the Chinese population. To explore the relationship between the *TCF7L2* gene polymorphism and T2DM in the Chinese population, we conducted a meta-analysis of 7990 subjects from ten studies. Depending on whether heterogeneity was present, we employed either a fixed or random-effects model, respectively, to assess the pooled odds ratio (ORs) and their corresponding 95% confidence interval (95% CI). *TCF7L2* rs12255372 G/T gene polymorphism was significantly associated with T2DM in the Chinese population under allelic (OR 1.38, 95% CI 1.20–1.60, $p = 8.59 \times 10^{-6}$), dominant (OR 1.67, 95% CI 1.20–2.32, $p = 0.002$), heterozygous (OR 1.65, 95% CI 1.18–2.31, $p = 0.003$), and additive genetic models (OR 1.53, 95% CI 1.13–2.07, $p = 0.006$). In the Chinese population, *TCF7L2* rs12255372 G/T gene polymorphism may correlate with an increased risk of T2DM. T allele of *TCF7L2* rs12255372 G/T gene polymorphism might confer a genetic risk for T2DM in the Chinese population.

Keywords *Transcription factor 7-like 2* · rs12255372 · Polymorphism · Type 2 diabetes mellitus

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Introduction

The Diabetes Atlas issued by the International Diabetes Federation at the 2015 World Diabetes Congress states that 415 million people suffer from diabetes mellitus (DM) worldwide. This means that one in 11 individuals has DM. From 2013, the number of patients with DM has increased by 31 million, and the IDF estimates that five million people died of DM in 2015. Given its international scale and tremendous scope, it is important to advance our knowledge of the complicated genetic and environment factors underpin this disease.

Transcription factor 7-like 2 protein (TCF7L2) is a member of the T cell transcription factor family that encodes for the glucagon promoter and also participates in the Wnt signal pathway. TCF7L2 regulates *glucagon* gene expression in intestinal endocrine cells, and thus plays an important role in maintaining plasma glucose homeostasis [1, 2]. The *TCF7L2* gene, located on the chromosome 10q25.3, spans 215.9 kb, and contains 14 exons [3]. In the European population, *TCF7L2* gene was demonstrated to be the most powerful gene associated with T2DM. In 2006, Grant et al. discovered rs12255372 in the third intron of the *TCF7L2* gene to be associated with T2DM [4].

Efforts to confirm this relationship in the Chinese population, however, has not been so clear. In 2009, FAN et al. found that the T allele of *TCF7L2* rs12255372 G/T gene polymorphism increased the T2DM risk in a Tianjin population [5]. On the other hand, Lu et al. found no such association between *TCF7L2* rs12255372 G/T gene polymorphism and T2DM in a Jiangsu population 2012 [6]. This result was replicated in a Chongqing population by Wang et al. in 2008 [7].

Here, we present our analysis of the data from 4072 T2DM patients and 3918 controls to evaluate the association of *TCF7L2* rs12255372 G/T gene polymorphism and T2DM in the Chinese population.

Table 1 Characteristics of the investigated studies of the association *transcription factor 7-like 2 (TCF7L2)* gene rs12255372 G/T polymorphism and type 2 diabetes mellitus (T2DM) in the Chinese population

Author	Year	Region	T2DM			Control			Matching criteria	Sample size (T2DM/control)
			GG	GT	TT	GG	GT	TT		
Ren Q [8]	2008	Beijing	490	9	0	490	9	0	Ethnicity	499/499
Wang ZH [7]	2008	Chongqing	430	15	0	296	8	0	Age, sex, ethnicity	445/304
Fan XW [5]	2009	Tianjin	287	65	0	171	5	0	Age, sex, ethnicity	352/176
Zhang MF [9]	2009	Xinjiang	31	12	1	33	12	2	Ethnicity	44/47
Zhang L [10]	2010	Hunan	219	17	0	206	12	0	Age, sex, ethnicity	236/218
Lu Q [6]	2012	Jiangsu	195	5	0	198	2	0	Age, ethnicity	200/200
Tu XJ [11]	2013	Zhejiang	290	20	0	514	29	0	Age, ethnicity	310/543
Zhang LW [12]	2014	Beijing	314	35	0	284	16	0	Age, sex, ethnicity	349/300
Yao H [13]	2015	Xinjiang	572	270	35	611	238	22	Age, sex, ethnicity, BMI	877/871
Chang YC [14]	2007	Taiwan	751	9	0	754	6	0	Ethnicity	760/760

PCR-RFLP and case-control study design were adopted in the above studies

T2DM type 2 diabetes mellitus, *BMI* body mass index, *PCR-RFLP* polymerase chain reaction-restriction fragment length polymorphism

Materials and methods

Publication search and inclusion criteria

The keywords, transcription factor 7-like 2, *TCF7L2,* rs12255372, T2DM, diabetic, and diabetes were used to search the following electronic databases: China National Knowledge Infrastructure, VIP database, Wanfang database, China Biological Medicine Database, and PubMed. The included studies were published between 2007 and 2015, with the latest update occurring on March 21, 2016).

The selected studies were retrieved in the light of the following inclusion criteria: (a) Assessment of the association of *TCF7L2* rs12255372 G/T gene polymorphism with T2DM;

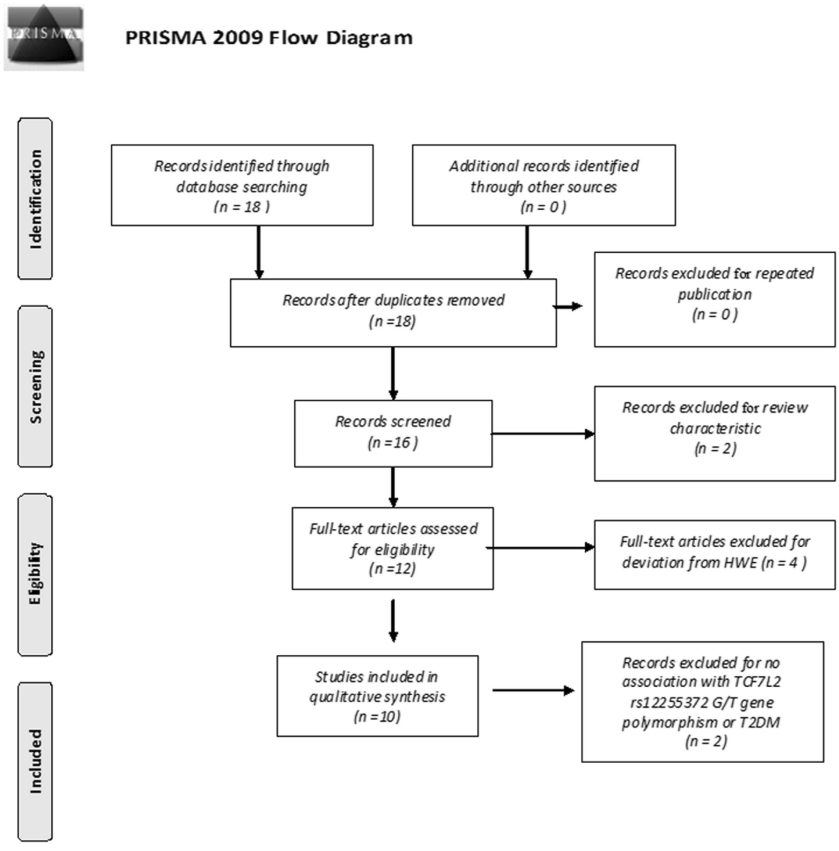

Fig. 1 PRISMA 2009 Flow diagram showing the schematic selection of studies for the meta analysis

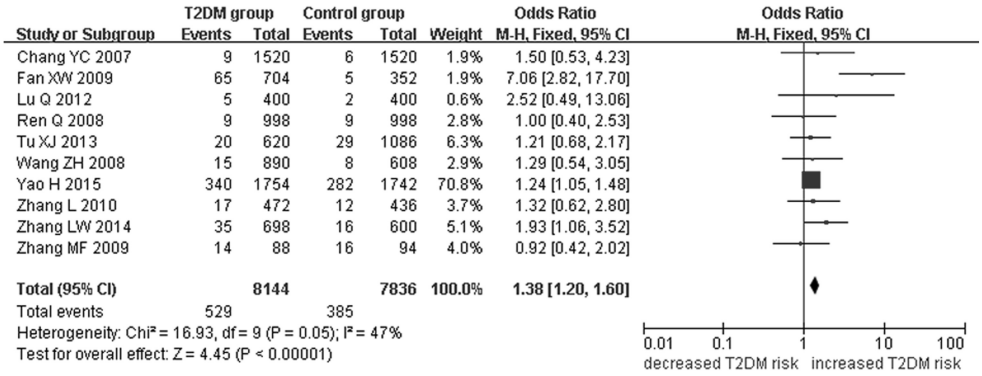

Fig. 2 Forest plot of T2DM associated with *TCF7L2* gene rs12255372 G/T under an allelic genetic model (distribution of T allelic frequency of *TCF7L2* gene)

(b) T2DM diagnosis and classification followed guidelines given by the World Health Organization in 1999. The T2DM patients should have the exact T2DM medical history. That is to say, fasting blood-glucose was no less than 7.0 mmol/L, and 2-h postprandial blood sugar was no less than 11.1 mmol/L. Conditions, such as type 1 diabetes mellitus, maternally inherited diabetes, acute and chronic complications of diabetes mellitus, hepatic and kidney function obstacle, ketosis, and other stress conditions were excluded. (c) Included studies were officially published case-control or cohort studies. (d) The control group genotype follows Hardy-Weinberg equilibrium (HWE).

Data extraction

Data was extracted by a standardized protocol by three investigators (Table 1). Two investigators took charge of finding duplicate studies, and the third served as the mediator to settle differences between the two. Studies that deviated from the major inclusion criteria, were published repeatedly, or provided inadequate data were excluded. Similar data sets found in different articles by a single author group was used once in our analysis. Items, such as the first author's name, publication year, region, matching criteria, genotype number, and total number of cases and controls, are listed in Table 1.

Statistical analysis

Revman 5.0 and STATA 12.0 software (StataCorp, College Station, TX) were used to perform the statistical analyses. Four genetic models, allelic (T allele distribution frequency), dominant (TG + TT vs. GG), heterozygous (TG vs. GG), and additive (total T vs. total G), were used in the present meta-analysis. The odds ratios (ORs) corresponding to its 95% confidence intervals (CIs) were used to compare the association of *TCF7L2* rs12255372 G/T gene polymorphism and T2DM. The Chi-square-based Q test was used to evaluate the heterogeneity between the individual studies with the significance set at $p < 0.05$ level [15]. If no heterogeneity existed among the studies, the fixed-effects model (the Mantel-Haenszel method) was used to estimate the pooled OR [16]. Otherwise, the random-effects model (DerSimonian and Laird method) was used [17]. The combined OR was determined by Z test with the significance was set at $p < 0.05$ level. HWE was assessed by Fisher's exact test with significance set at $p < 0.05$ level. Funnel plot evaluated potential publication bias. Egger's linear regression test on the natural logarithm

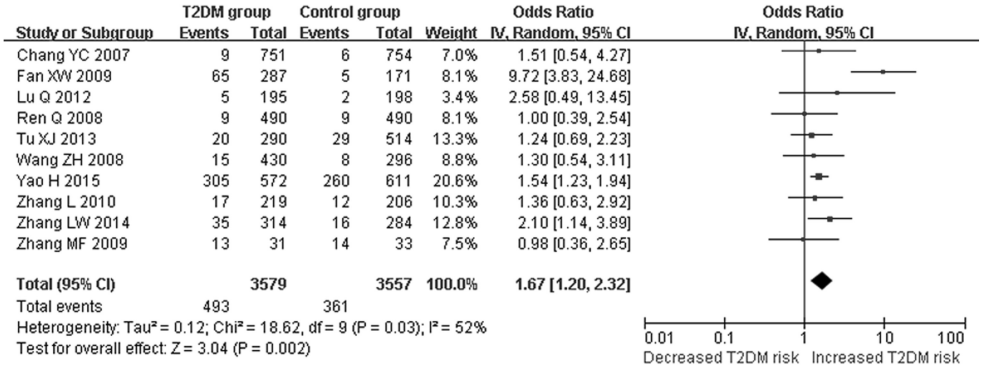

Fig. 3 Forest plot of T2DM associated with *TCF7L2* gene rs12255372 G/T gene polymorphism under a dominant genetic model (TG + TT vs. GG)

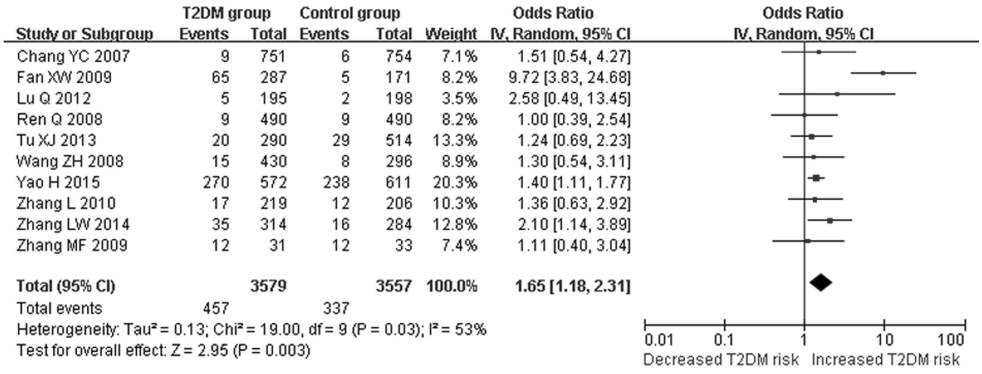

Fig. 4 Forest plot of T2DM associated with *TCF7L2* gene rs12255372 G/T gene polymorphism under a heterozygous genetic model (TG vs. GG)

scale of the OR was adopted to assess the funnel plot symmetry with significance set at $p < 0.05$ level [18].

Results

Studies and populations

Of the 18 papers found in our initial literature search, ten met the inclusion criteria. Among the eight excluded studies, four studies were rejected for controls violating the HWE [19–22]. Two studies were of review character, and another two studies had nothing to do with T2DM or *TCF7L2* rs12255372 G/T gene polymorphism. The total data were abstracted from 4072 T2DM patients and 3918 controls (Fig. 1, Table 1) [5–14]. Eight provinces were represented in the meta-analysis: Beijing, Chongqing, Tianjin, Xinjiang, Hunan, Jiangsu, Zhejiang, and Taiwan.

Pooled analyses

TCF7L2 rs12255372 G/T gene polymorphism was significantly associated with T2DM in the Chinese population under allelic (OR 1.38, 95% CI 1.20–1.60, $p = 8.59 \times 10^{-6}$), dominant (OR 1.67, 95% CI 1.20–2.32, $p = 0.002$), heterozygous (OR 1.65, 95% CI 1.18–2.31, $p = 0.003$), and additive genetic models (OR 1.53, 95% CI 1.13–2.07, $p = 0.006$). No heterogeneity existed under the allelic genetic model ($p_{\text{heterogeneity}} = 0.05$, $I^2 = 47.0\%$). (Figs. 2, 3, 4, and 5, Table 2).

As the heterogeneity existed under the dominant, heterozygous, and additive genetic models (dominant $p_{\text{heterogeneity}} = 0.03$, $I^2 = 52.0\%$; heterozygous $p_{\text{heterogeneity}} = 0.03$, $I^2 = 53.0\%$; additive $p_{\text{heterogeneity}} = 0.04$, $I^2 = 48.0\%$), we performed a meta-regression to identify the source of that heterogeneity, which confirmed TG genotype number in the T2DM group (TG1) was the main heterogeneity source ($p < 0.05$). We then conducted a subgroup analysis stratified by the TG1 under the three genetic models. In the subgroup 1, which consisted of TG1 < 16, we found no significant association between *TCF7L2* rs12255372 G/T gene polymorphism and T2DM under dominant, heterozygous, and additive genetic models. (dominant OR 1.25, 95% CI 0.79–1.97, $p = 0.34$; heterozygous OR 1.29, 95% CI 0.82–2.04, $p = 0.27$; additive OR 1.19, 95% CI 0.78–1.83, $p = 0.42$). No heterogeneity was detected in subgroup 1 ($p > 0.05$, $I^2 = 0\%$). In subgroup 2, where TG1 > 16, a significant association between *TCF7L2* rs12255372 G/T gene polymorphism and T2DM was detected under the three genetic models (dominant OR 2.01, 95% CI 1.21–3.33, $p = 0.007$; heterozygous OR 1.68, 95% CI 1.39–2.02, $p = 6.30 \times 10^{-8}$; additive OR 1.81, 95% CI 1.13–2.89, $p = 0.01$). In the subgroup 2, the heterogeneity was even much stronger than that in the whole population (dominant $p_{\text{heterogeneity}} = 0.003$, $I^2 = 75.0\%$; heterozygous $p_{\text{heterogeneity}} = 0.001$, $I^2 = 78.0\%$; additive $p_{\text{heterogeneity}} = 0.004$, $I^2 = 74.0\%$). (Table 2).

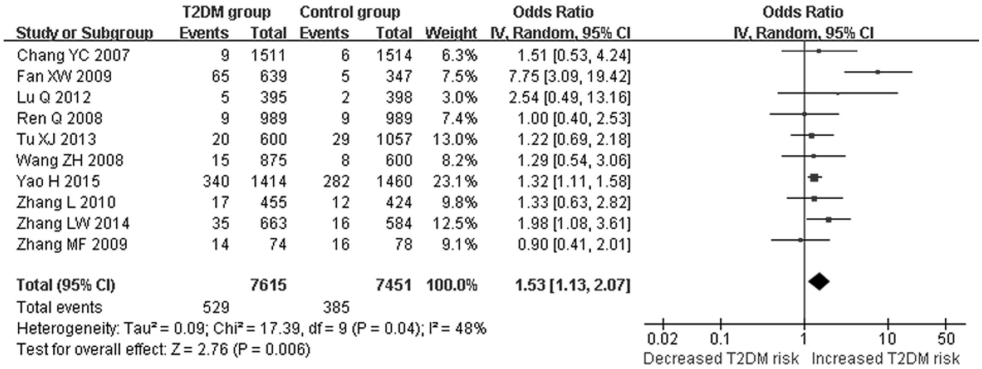

Fig. 5 Forest plot of T2DM associated with *TCF7L2* gene rs12255372 G/T gene polymorphism under an additives genetic model (T vs. G)

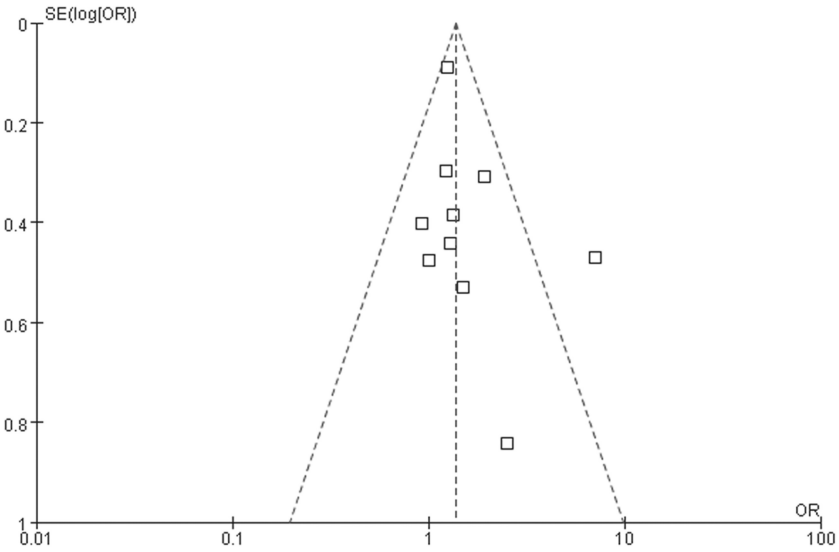

Fig. 6 Funnel plot for studies of the association of T2DM associated and *TCF7L2* gene rs12255372 G/T gene polymorphism under an allelic genetic model (distribution of T allelic frequency of *TCF7L2* gene) The *horizontal* and *vertical axis* correspond to the OR and confidence limits. *OR* odds ratio, *SE* standard error

Bias diagnostics

The Egger's test and funnel plot were adopted to evaluate the publication bias of the separate studies. Egger's test result suggested that under the allelic genetic model, no significant publication bias existed in the present meta-analysis ($T = 0.20$, $p = 0.848$). In addition, not any visual publication bias was observed in the funnel plot under the allelic genetic model (Fig. 6).

Discussion

In this meta-analysis, a significant association was found between T2DM and *TCF7L2* rs12255372 G/T gene polymorphism in the Chinese population under the allelic (OR 1.38), dominant (OR 1.67), heterozygous (OR 1.65), and additive (OR 1.53) genetic models. No heterogeneity existed under the allelic genetic model. Heterogeneity present in the remaining genetic models pushed us to use the random effects model in the current meta-analysis. Moreover, TG1 was verified to be the heterogeneity source by a meta-regression ($p < 0.05$) and the subgroup analysis stratified by TG1 further confirmed our result. Regarding the additive genetic model, in this study, all subjects carrying T allele have been compared with all subjects carrying G allele, and the OR was 1.53. It was indicated that the T2DM risk conferred by T allele was increased by 1.53-fold to that by G allele. Therefore, it was concluded that the T allele of *TCF7L2* rs12255372 G/T gene polymorphism conferred a genetic risk for T2DM in the Chinese population.

DM is a dysmetabolic syndrome characterized by hyperglycemia caused by various etiologies. T2DM is a complicated polygenic disease. The genetic variants and environmental

Table 2 Summary of meta-analysis of association between *TCF7L2* gene rs12255372 G/T polymorphism and T2DM in the Chinese population

Genetic model	Pooled OR (95% CI)	*Z* value	*p* value	Study number	T2DM size	Control size	$P_{\text{heterogeneity}}$ (I^2 %)
Allelic genetic model	1.38 (1.20–1.60)	4.45	8.59×10^{-6}	10	4072	3918	0.05 (47.0%)
Dominant genetic model	1.67 (1.20–2.32)	3.04	0.002	10	4072	3918	0.03 (52.0%)
Subgroup 1 TG1 <16	1.25 (0.79–1.97)	0.95	0.34	5	1948	1810	0.86 (0%)
Subgroup 2 TG1 >16	2.01 (1.21–3.33)	2.70	0.007	5	2124	2108	0.003 (75.0%)
Heterozygous genetic model	1.65 (1.18–2.31)	2.95	0.003	10	4072	3918	0.03 (53.0%)
Subgroup 1 TG1 <16	1.29 (0.82–2.04)	1.10	0.27	5	1948	1810	0.86 (0%)
Subgroup 2 TG1 >16	1.68 (1.39–2.02)	5.41	6.30×10^{-8}	5	2124	2108	0.001 (78.0%)
Additive genetic model	1.53 (1.13–2.07)	2.76	0.006	10	4072	3918	0.04 (48.0%)
Subgroup 1 TG1 <16	1.19 (0.78–1.83)	0.81	0.42	5	1948	1810	0.80 (0%)
Subgroup 2 TG1 >16	1.81 (1.13–2.89)	2.46	0.01	5	2124	2108	0.004 (74.0%)

CI confidence interval, *OR* odds ratio, *T2DM* size the total number of T2DM cases, *control size* the total number of control group, *Allelic genetic model* T allele distribution frequency, *Dominant genetic mode*: TG + TT vs. GG, *Heterozygous genetic model* TG vs. GG, *Additive genetic model* T vs. G

*$P \leq 0.05$

factors contribute the T2DM ultimately. Factors that contributed to the international scope of DM are just as multifaceted. Economic development, changes in diet, reduced levels of physical activities, increased prevalence of obesity, and the prolongation of life have all contributed to increasing T2DM-associated morbidity to epidemic levels.

The two main mechanisms for T2DM pathogenesis are insulin resistance (IR) and pancreatic β cells dysfunction, both of which are affected by the *TCF7L2* gene. This gene encodes the T cell transcriptional factor-4 (TCF-4) which plays a central role in the Wnt signaling pathway in intestinal and pancreatic tissues. Wnt stimulation causes translocation of beta-catenin into the nucleus where it interacts with TCF-4 to modify transcriptional activity. Through this mechanism, TCF-4 can regulate the proliferation of pancreatic island â cells and insulin secretion. In addition, TCF-4 could influence the glucagon-like peptide-1 (GLP-1) synthesis in the small intestinal L cells [23]. Variations in the *TCF7L2* gene could also disturb adipose formation and adipocyte function by altering CEBPα (CCAAT enhancer-binding protein α) and peroxisome proliferators-activated receptor γ (PPAR2 γ). This can lead to triacylglycerol deposition in the peripheral tissues, such as liver and muscle, and promotes IR [24].

In 2006, Cauchi et al. found T allele of *TCF7L2* rs12255372 G/T gene polymorphism to reduce both insulin secretion and insulin sensitivity to glucose [25]. The prospective study showed that individuals with the T allele of *TCF7L2* rs12255372 G/T gene polymorphism had elevated levels of glucose during 2-h post-meal and during fasting. These individuals also had a relatively low boost insulin production index under basic conditions. These factors made them more susceptible to T2DM [24, 26]. This conclusion was confirmed in the current meta-analysis.

In 2013, Dou et al. have conducted a meta-analysis on the relationship between T2DM and *TCF7L2* rs12255372 G/T gene polymorphism in the Chinese population where they concluded the gene polymorphism had no significant effect on T2DM susceptibility ($p > 0.05$) [27]. This analysis consisted of five individual studies and 4251 subjects. The larger sample size of this meta-analysis should present a more objective conclusion than that of Dou. Furthermore, in Dou's meta-analysis, the subgroups analysis of the studies based on age, sex, and BMI was not carried out due to their insufficient studies. In the current meta-analysis, the meta-regression analysis has shown that the heterogeneity of the included studies was not associated with the matching of study subjects for age, gender, or ethnicity.

This study is not without limitations. Many factors besides *TCF7L2* rs12255372 G/T gene polymorphism can influence plasma TCF-4 levels, such as *TCF7L2* rs11196218 G/A, rs7903146 C/T, and rs290487 T/C gene polymorphisms, smoke, diet habit, and hypertension [27, 28]. Much remains to be learned on how this gene polymorphism affects patient susceptibility to DM and this requires large-scale studies on the subject.

In short, in the current meta-analysis, a significant association was detected between *TCF7L2* rs12255372 G/T gene polymorphism and T2DM risk in the Chinese population. The individuals with the T allele of *TCF7L2* rs12255372 G/T gene polymorphism may be more susceptible to T2DM in the Chinese population. This conclusion might guide us to prevent T2DM by formulating an individual treatment strategy for the Chinese population. Considering the above-mentioned limitations, more large-scale studies should be conducted to confirm this conclusion in the future.

Acknowledgements This work was funded by the National Natural Science Foundation of China (NSFC 81100073 to Dr. Yan-yan Li), Excellent Young and Middle-Aged Teachers Assistance Program of Nanjing Medical University for Dr. Yan-yan Li (2013–2015, JX2161015034), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We thank all our colleagues working in the Department of Geriatrics, the First Affiliated Hospital of Nanjing Medical University.

Authors' contributions Conceived and designed the experiments: Yan-yan Li. Performed the experiments: Yan-yan Li, Ge Gong, and Hong-yu Geng. Analyzed the data: Yan-yan Li and Xin-xing Yang. Contributed reagents/material/analysis tools: Yan-yan Li.Wrote the manuscript: Yan-yan Li. Reference collection and data management: Yan-yan Li. Statistical analyses and paper writing: Yan-yan Li. Study design: Yan-yan Li.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):62–68
DOI 10.1007/s13410-016-0544-z

ORIGINAL ARTICLE

Paraoxonase 1 (PON1) gene polymorphism and haplotype analysis in type 2 diabetes mellitus: a case–control study in the south Iranian population

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Received: 29 August 2016 / Accepted: 19 December 2016 / Published online: 9 January 2017

Abstract Paraoxonase-1 (PON1) is involved in prevention of lipid peroxidation and has been associated with diseases characterized by high oxidative stress, such as cardiovascular disease and diabetes. It has been studied as a potential candidate gene for diabetes risk, but direct evidence from genetic association studies remains inconclusive. We performed an analysis in order to investigate the association between three PON1 polymorphisms (Q192R, L55M, and −108C>T) with type 2 diabetes mellitus (T2DM) in south Iranian population. A total of 340 individuals (171 documented T2DM patients and 169 healthy controls) were examined in this study. After DNA isolation and PCR-RFLP, the products were analyzed for L55M and Q192R polymorphisms in coding region and for −108C>T in promotor sequence of PON1. Statistical analysis showed that all genotypes of Q192R and −108C>T polymorphisms were not associated with diabetes ($P > 0.05$). However, in PON1 55 gene polymorphism, the allelic frequency of methionine (M) was significantly higher in T2DM patients compared to controls (37 vs. 28%, $P < 0.05$). In addition, a strong association was found between the LM+MM group and diabetes ($P = 0.006$). The results of the haplotype analysis for the combinations of the three polymorphisms in patients were shown that the haplotype L-T-R frequency was significantly lower in patients as compared to the controls with odds ratio of 0.28 (OR = 0.28, 95% CI 0.13–0.60, $P = 0.0004$). The most frequent haplotype in two patient and control groups was L-C-Q (31 and 30.2%, respectively). Met allele of PON1 55 gene polymorphism is an independent risk factor for T2DM and the L-T-R may represent as a protective haplotype in south Iranian population. Our results showed that the frequencies of polymorphisms of PON1 in the south Iranian population differ to some of those observed in other ethnic groups and provide useful data for epidemiological studies.

Keywords Paraoxonase (PON) · Genetic polymorphism · PCR-RFLP · Type 2 diabetes mellitus

Introduction

Diabetes mellitus (DM) is a complex and chronic metabolic disorder characterized by metabolic abnormalities and long-term complications involving the eyes, kidneys, nerves, and blood vessels [1, 2]. The prevalence of diabetes mellitus is increasing steadily all over the world and it is estimated that the number of people with DM worldwide exceeds to 336 million in 2011, which most of them have type 2 diabetes mellitus (T2DM). North of the Africa and Middle East have shown the highest regional prevalence for 2011 and Iran was among the greatest diabetic prevalence of Middle East [3] (http://www.emro.who.int/ncd/pdf/stepwise_saa_05.pdf), (http://www.who.int/chp/steps/2007STEPSreportIran.pdf).

DM is a major risk factor for the development of coronary artery disease (CAD), which is one of the leading causes of morbidity and mortality in developed countries. It is also associated with increased production of reactive oxygen species (ROS), glycation, and glycol-oxidation of low-density lipoprotein (LDL) by glucose. These conditions may cause increased serum oxidative stress which plays an important role

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in the reduction of plasma antioxidant defenses and development of diabetes and its complications [4–8].

Paraoxonase (PON) is a member of the Ca^{+2}-dependent ester hydrolase family which has central role in a wide variety of human illnesses such as cardiovascular disease, diabetes mellitus, metabolic syndrome, obesity, non-alcoholic steatohepatitis, and several mental disorders. This antioxidant enzyme is being used up in balancing the oxidative stress in diabetes mellitus and hence a decrease of PON1 activity has been observed in diabetic patients [9].

Human serum paraoxonase 1 (PON1. EC 3.1.8.1.) is a 43-kDa enzyme which possess antioxidant and antiatherogenic properties through its ability to hydrolyze paraoxon, the active toxic metabolite of insecticides and nerve agents and closely associated with high-density lipoprotein (HDL) particles in human plasma. It has been shown to prevent the accumulation of lipid oxidation products (lipoperoxides) on LDL and inhibit the transformation of LDL into atherogenic particles, thereby prevents the induction of monocyte-endothelial interactions on the arterial wall [10–14]. PON1 plays a crucial role in regulating antioxidant defenses and inflammatory response in the vascular milieu. It represents a biomarker reflecting HDL function and as such, a better monitor of cardiovascular risk. PON1 activity is lowered in vascular diseases and PON1 knockout mice have increased susceptibility to atherosclerosis, supporting the role of PON1 as an antioxidant and antiatherogenic enzyme in several studies [15, 16].

PON1 also has the ability to stimulate the production of insulin and glucose transport protein 4 (Glut 4). It substantially attenuates diabetes development via its ability to stimulate pancreatic β-cell insulin production and secretion and its unique antioxidative characteristics. Enzymatic activity of PON1 is found to be decreased in diabetic patients in parallel to the duration of diabetes and, this is associated with the acceleration of vascular wall impairment and cardiovascular disease development in diabetic patients [17–19].

PON family of the enzymes consists of three members, PON1, PON2, and PON3 which are located on chromosome 7 and share approximately 65% similarity at amino acid level [21]. PON1 is produced mainly in the liver and encoded by the polymorphic gene on chromosome 7q21.3 [20–22]. Gene polymorphisms have potent influences on PON1 enzymatic activities. The polymorphism characterized in paraoxonase activity polymorphisms consisted of missense mutations in the coding region of PON1, resulting in a glutamine (Q)/arginine (R) substitution at codon 192 [23–25], and in amino acid substitution at position 55 Leu (L)/Met (M), respectively. The PON1 gene has nearly 200 reported SNPs (single nucleotide polymorphisms) which Q192R (db SNP rs. 662) and L55M (db SNP rs. 854560) substitutions are the most commonly studied polymorphisms in this group [24].

The PON1R192 hydrolyzes paraoxon much more efficiently than does PON1Q192, while in the case of sardine, soman and at metabolizing oxidized HDL or LDL, PON1Q192 alloform functions more efficiently than PON1R192 [26]. PON1L55M is another coding region polymorphism, resulting in amino acid substitution at position 55 Leu (L)/Met (M) and it is associated with plasma PON1 protein levels. In spite of low enzyme activities in PON1M55 genotype, PON1L55 has been associated with high plasma PON1 level.

Different polymorphisms are reported in the promoter gene of PON1 that led to the discovery of various degrees of influence over this gene expression; physiologically, it has been shown that there is a good correlation between the type of variation and difference in serum PON1 concentration and activity [27, 28]. One of these polymorphisms is located at position −108 (C/T) (db SNP rs. 705379). This site appears to be the most significant contributor to PON1 serum variation. The C-108T substitution has the most significant effect on plasma PON1 levels. The –108C allele provides about twice higher activities of PON1than those seen with the –108T allele [21]. Moreover, it is observed that there is a positive association between the low activity of T allele and vascular disease in young adults and in patients with T2DM [29, 30]. In the current study, we evaluated the association of three implied single nucleotide polymorphisms (SNPs) in the PON 1 gene with T2DM. To our knowledge, the current study is the first investigation done on Iranian diabetic patients

Material and methods

Subjects

This case–control study was performed in Shiraz, Iran during 1 year. Eligible participants were 340 unrelated individuals who were consisted of 171 patients including 111 females and 60 males aging 25–64 years with T2DM documented by clinical tests and 169 healthy control individuals, including 111 females and 58 males aging 25–64 years. In our study, subjects were included in diabetic group if they had a fasting glucose concentration ≥126 mg/dL or glucose concentration ≥200 mg/dL 2 h after a 75-g oral glucose tolerance test. Moreover, patients with type 1 diabetes, pregnant or lactating women, smokers and alcoholics were excluded from our study (Table 1). All patients and controls were recruited from Shahid Motahhari outpatients, affiliated to Shiraz University of Medical Sciences. In addition, all procedures were in accordance with the ethical standards of the Shiraz university of Medical Science institution and with the 1964 Helsinki declaration. Prior to the commencement of the research, participants' consent was obtained from each individual.

DNA isolation and PON1 genotyping analysis

Two-milliliter venous blood samples were obtained from each individual and collected into EDTA-containing tubes. Genomic DNA was extracted from whole blood by

Table 1 Demographic and clinical characteristics of the study

Value	T2DM patients (n = 171)	Control (n = 169)	P
Age (years)	47 ± 8.3	45.9 ± 10.3	0.32
Sex (female/male)	117/54	114/55	0.88
Fasting blood glucose (mg/dL)	194.7 ± 59.1	90.4 ± 6.7	<0.001*
HbA1c (%)	7.7 ± 2.0	5.3 ± 0.3	<0.001*
BMI (kg/m^2)	26.8 ± 4.4	26.7 ± 3.8	0.93
Cholesterol (mg/dL)	202.7 ± 52.7	176.5 ± 35.3	<0.001*
Triglyceride (mg/dL)	180.1 ± 85.6	129.9 ± 53.6	<0.001*
HDL (mg/dL)	45.8 ± 9.8	46.4 ± 9.1	0.54
LDL (mg/dL)	122.5 ± 45.9	106.1 ± 28.9	<0.001*

*Statistically significant

Cinnagen Kit DNP™ protocol (Sinagene Company, Tehran, Iran) and PCR was performed for amplification of PON1 genes using the mentioned primers (Table 2). SNPs were identified by PCR-RFLP analysis as described by Grdic et al. [31]. The nucleotide substitution corresponding to position 192(Gln/Arg) and 55(Met/Leu) creates a BspPI (Fermentas Life Science) and Hin1II (Fermentas Life Science) restriction site. Polymorphisms in promotor region of PON1 gene –108C>T was studied using restriction endonuclease Bsr BI (Fermentas Life Science) (Table 2). In the final step, the digested fragments were electrophoresed in 3% agarose gel.

Statistical analysis

Statistical analysis was performed using version 16 of SPSS software (SPSS, Inc., Chicago, IL, USA). Comparisons between categorical variables were done using Chi-square (χ^2) test and the relative associations between PON1 genotypes and the risk of developing T2DM was estimated by using conditional logistic regression for calculation of odd ratios (ORs) and 95% confidence interval (CIs). All P values were two-sided and a probability of $P < 0.05$ was considered statistically significant. Logistic regression was also used to analyze age, sex, HbA1c, fasting blood glucose (FBS), and BMI (Table 1). The frequencies of all genotypes and alleles in our population were compared with those in other populations. Moreover, The Hardy–Weinberg equilibrium was carried out in Arlequin (Version 3.5.1.2) software. The haplotype frequency was also determined by Arlequin (Version 3.5.1.2) software. Fisher's exact test was used to find differences in haplotype frequency between patients and controls.

Results

Demographic characteristics and biochemistry parameters of the subjects enrolled in the study are shown in Table 1. Our results revealed no correlation between age, sex, body mass index (BMI), and HDL level in the T2DM and control groups ($P > 0.05$), but there was a significant difference between patient and control groups in FBS, HbA1c, cholesterol, triglyceride (TG), and LDL levels ($P < 0.001$) (Table 1).

The allele frequencies of the PON1 polymorphisms along with the frequency distribution of PON1 192, PON1 55, and PON1−108C>T genotypes were shown in Table 3. The genotypic distribution of the studied polymorphisms was in Hardy–Weinberg equilibrium (HWE) in both groups.

As regards PON1 192 genotypes distributions and allele frequencies in the studied groups, the QQ genotype (Gln/Gln) was the most common in diabetic patients (70.17%) as well as in the controls (62.13%), while the RR genotype (Arg/Arg) was the lowest one in both (1.75, 1.77%), respectively. Significant difference was not observed among the genotypes of Q192R polymorphism in diabetic patients compared to the controls ($P > 0.05$). The allelic frequency of glycine192 (Q) was higher in the diabetic patients than controls (84 vs. 80%), but no significant difference between the allele frequencies for the PON1 192 polymorphism was observed in diabetic patients as compared to the controls ($P > 0.05$). Regarding the PON1 55 polymorphism, the LL genotype (Leu/Leu) was the most common in the controls (49.70%), whereas the LM (56.72%) was more common than the LL genotype in diabetic patients. The genotype distribution of PON1 55 polymorphisms in diabetic

Table 2 Primers used for PON1 polymorphisms typing

SNP	Primer (5′ → 3′)	Annealing temperature (°C)	Amplicon (bp)	Restriction enzyme	RFLP fragments (bp)
Q192R	F: TATTGTTGCTGTGGGACCTGAG	63	238	*Bsp*PI	Q allele: 238
	R: CCTGAGAATCTGAGTAAATCCACT				R allele: 175 + 63
L55M	F: CCTGCAATAATATGAAACAACCTG	56	172	*Hin*1II	L allele: 172
	R: TGAAAGACTTAAACTGCCAGTC				M allele: 103 + 69
−108C>T	F: AGCTAGCTGCCGACCCGGCGGGGAGG aG	70	240	*Bsr*BI	C allele: 212 + 28
	R: GGCTGCAGCCCTCACCACAACCC				T allele: 240

Table 3 PON1 genotype distribution in controls and patients with type 2 diabetes

SNP	Genotype	Patients (171)	Control (169)	P^a	OR	95% CI	P^b
Q192R	QQ	120 (70.17)	105 (62.13)	0.136	1 (reference)	–	–
	QR	48 (28.07)	61 (36.09)	0.131	0.68	0.43–1.09	0.689
	RR	3 (1.75)	3 (1.77)	1.00	0.87	0.17–4.42	0.875
	QR+RR	51 (29.82)	64 (37.87)	0.136	0.69	0.44–1.09	0.118
	Allele Q	288 (0.84)	271 (0.80)		1 (reference)	–	–
	Frequency R	54 (0.16)	67 (0.20)		0.76	0.50–1.15	0.202
L55M	LL	59 (34.50)	84 (49.70)	0.06	1 (reference)	–	–
	LM	97 (56.72)	76 (44.97)	0.039	1.81	1.16–2.84	0.09
	MM	15 (8.77)	9 (5.33)	0.290	2.37	0.97–5.78	0.057
	LM+MM	112 (65.50)	85 (50.30)	0.006	1.87	1.21–2.90	0.005*
	Allele L	215 (0.63)	244 (0.72)		1 (reference)	–	–
	Frequency M	127 (0.37)	94 (0.28)		1.53	1.10–2.15	0.011*
−108C>T	TT	69 (40.35)	72 (42.60)	0.741	1 (reference)	–	–
	CT	61 (35.67)	66 (39.05)	0.575	0.96	0.59–1.55	0.882
	CC	41 (23.98)	31 (18.34)	0.233	1.38	0.77–2.44	0.896
	CT+CC	102 (59.65)	97 (57.40)	0.741	1.09	0.71–1.69	0.673
	Allele C	199 (0.58)	210 (0.62)		1 (reference)	–	–
	Frequency T	143 (0.42)	128 (0.38)		1.18	0.86–1.62	0.331

*Statistically significant

[a] Pearson's χ^2 test for respectively genotypic distributions

[b] Binary logistic regression test

patients was statistically significant different compared to the controls. Frequencies of LM+MM genotypes of L55M polymorphism were significantly higher in patients as compared to the controls ($P < 0.05$) and were associated with T2DM with odds ratios of 1.87 (OR 1.87, 95% CI 1.21–2.90). The allelic frequency of methionine 55 (M) was higher in the diabetic group than controls (37 vs. 28%) and statistically significant difference between controls and diabetic patients was detected ($P < 0.05$) (Table 3). The genotypes and allele frequencies of promoter −108C/T polymorphisms were not significantly different in both groups ($P > 0.05$).

Haplotype frequency of coding Q192R, L55M and non-coding −108C>T polymorphisms among control and diabetic groups are shown in Table 4. The results of the haplotype analysis for the combinations of the three polymorphisms in patients and controls are shown in Fig. 1. The most frequent haplotype in two patient and control groups was L-C-Q (31, 30.2%, respectively). The haplotype L-T-R frequency was significantly lower in patients as compared to the controls with odds ratio of 0.28 (OR = 0.28, 95% CI 0.13–0.60, $P = 0.0004$).

Discussion

Diabetes is correlated with a high level of endogenous inflammatory, oxidative stress, and increased susceptibility to

Table 4 PON1 coding (Q192R, L55M) and promoter (−108C/T) haplotype frequency in controls and patients with type 2 diabetes

Haplotype	Haplotype frequency in patients (171)	Haplotype frequency in controls (169)	OR	95% CI	*P* value
LTQ	0.260	0.290	0.90	0.64–1.26	0.56
MTQ	0.213	0.163	1.31	0.96–1.34	0.19
LCQ	0.310	0.302	1.03	0.74–1.42	0.92
LCR	0.029	0.026	1.10	0.41–2.98	0.97
LTR	0.029	0.104	0.28	0.13–0.60	0.0004*
MCQ	0.058	0.047	1.24	0.60–2.55	0.65
MTR	0.076	0.059	1.28	0.68–2.45	0.50
MCR	0.023	0.008	0.37	0.09–1.44	0.22

OR odds ratio, *CI* confidence interval from conditional logistic regression

*Statistically significant

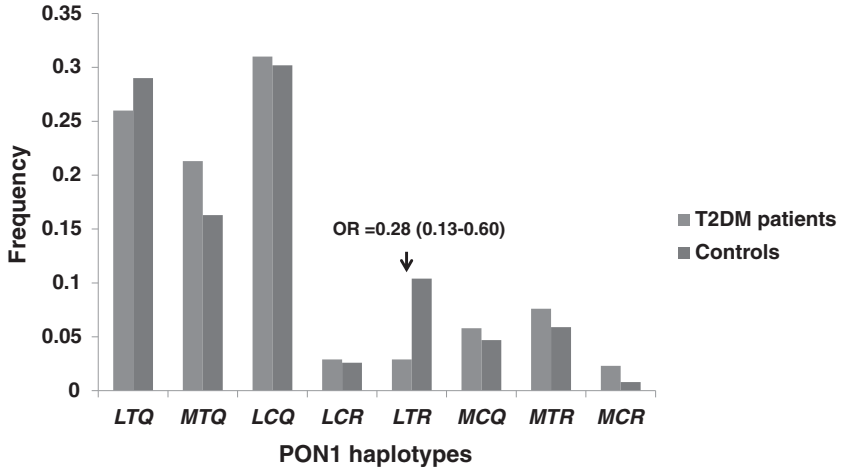

Fig. 1 PON1 haplotype frequencies in controls and T2DM patients. The haplotype L-T-R frequency was significantly lower in patients as compared to the controls ($P < 0.05$). Order of SNPs is L55M (rs 854560), −108C>T (rs 705379), and Q192R (rs 662)

vascular diseases [32] and characterized as a multifactorial and polygenic disease [32]. Hereupon, identifying the genes and their variants that contribute to the pathogenesis of diabetes has been an important and challenging topic during decades. Investigation of the single nucleotide polymorphisms of antioxidant enzymes which account for elimination of toxic intermediates will help us to understand the genetic etiology of diabetes [33]. The current biochemical and genetic studies illustrate that the PON1, a HDL-associated protein, promulgate remarkable systemic antioxidant effects in humans [34]. Polymorphisms within PON1 gene will independently affect PON1 activity and are defined as PON1 Q192R, L55M and −108C>T [35]. This study was done in continuum of our previous studies regarding the association between polymorphisms of glutathione-s-transferase (GST) M1 and T1 genes and the risk of diabetes; it was concluded that only GSTM1-null genotype is associated with diabetes [32, 33]. Therefore, in this study, we investigated the association between polymorphisms of coding (Q192R and L55M) and promoter (−108C>T) regions of PON1 gene and risk of diabetes in the south of Iran. Our results regarding mono-allelic analysis of L55M polymorphism indicated that L allele had a regressive effect and M allele had an augmentative effect on development of diabetes. The M allele is significantly more frequent in diabetic patients as compared to the controls with an odds ratio of 1.53 (OR = 1.53, 95% CI 1.10–2.15, $P = 0.011$).

Till date, no data is available in Iran regarding association of PON1 haplotypes with T2DM. The most frequent haplotype in two patient and control groups was L-C-Q and L-T-R haplotype was significantly lower in patients as compared to the controls. The results of the haplotype analysis for the combinations of the three polymorphisms in patients were shown that the L-T-R may represent as a protective haplotype in south Iranian population.

Various studies with different outcomes have been conducted in some populations to discover PON1 polymorphisms. Our findings are consistent with the results of B. Mackness et al. and B. Agachan et al. who found no significant difference in genotype distribution for Q192R, L55M and −108C>T polymorphism in T2DM patients and controls [36, 37]. On the other hand, the results of our study are opposed to N. Gupta et al. who found that QR, RR (Q192R) genotypes were more common in patients as compared to the controls [38]. They also found that R allele is associated with diabetes development and there is not any association between L55M polymorphism alleles and development of the disease. Regarding the −108C>T polymorphism, their results showed minor effect of this polymorphism on diabetes development.

Our results are also in agreement with M. Flekač et al. report from Czech Republic which revealed that R allele (from Q192R polymorphism) is less frequent in patients than in the controls with synergistic effect between Q and R alleles to reduce diabetes development and M allele (from L55M polymorphism) is more frequent in the diabetes group than the control group with augmentative effect between M alleles on development of diabetes [39]. Also, they found that −108C>T polymorphism has a neutral effect on diabetes development which is in agreement with our results, indicating minor effect of this polymorphism on diabetes development [39].

Many researchers concluded that the LL (L55M) and RR (Q192R) genotypes of PON1 gene are associated with higher levels of PON1 activity than MM and QQ genotypes. This phenomenon may be more protective against lipid peroxidation [38, 39]. In addition, widespread presence of MM and QQ genotypes in diabetes is correlated with lower glucose control [38]. These evidences support our data that PON1 55M variant was associated with disease susceptibility and MM and QQ genotypes are more common in diabetic patients. However, further studies are needed to clarify whether paraoxonase is involved in diabetes by preventing lipid oxidation

and whether there are other mechanisms linking paraoxonase to T2DM. A limitation of our study should be taken into consideration. We did not measure paraoxonase activity. However, doing so might not have clarified the complex causal relationship between paraoxonase activity and diabetes. Moreover, paraoxonase levels in serum might not correlate with lipoprotein-bound paraoxonase.

T2DM is a multifactorial disease influenced by complex genetic as well as environmental factors [40]. The conflicting results obtained by studies on different populations might be attributed to the interaction of several factors, such as different lifestyle habits (including physical activity, food habit, stress, smoking, etc.), differences in ethnic background of each population, different kinds of environment, and differential susceptibility to diseases. Finally, genetic context may be rather correlated with diabetes control and as a result, enzyme acts against oxidative stress [36] and this could be a reason for lack of agreement in different studies.

Conclusion

In conclusion, the results of the present study provide evidence that the PON1 55M allele is independently associated with an increased risk of T2DM in the south Iranian population. Moreover, the results of the haplotype analysis for the combinations of the three polymorphisms in patients were shown that the L-T-R may represent as a protective haplotype in this population. Our results showed that the frequencies of polymorphisms of PON1 in the south Iranian population differ to some of those observed in other ethnic groups and provide useful data for epidemiological studies.

Acknowledgments The authors would like to gratefully thank the staff of clinical diagnostic laboratory of Shahid Motahhari outpatient at Shiraz University of Medical Sciences. This work is the result of a research project supported by Shiraz University of Medical Sciences.

Compliance with ethical standards

Funding This study was funded by Transplant Research Center affiliated to Shiraz university of Medical Science, Iran (grant number 160-92).

Conflict of interest The authors declare that they have no conflict of interest.

Human ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Shiraz university of Medical Science institution and with the 1964 Helsinki declaration.

This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all participants included in the study.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):69–74
DOI 10.1007/s13410-017-0548-3

ORIGINAL ARTICLE

Prevention of type 2 diabetes mellitus by changes in diet among subjects with abnormal glucose metabolism: a randomized clinical trial

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Received: 19 July 2016 / Accepted: 7 January 2017 / Published online: 14 January 2017

Abstract Previous studies demonstrated that changes in lifestyle slow the progression of impaired glucose tolerance to overt diabetes but few trials examined the effect of diet alone without weight loss on the prevention of diabetes. We investigated the efficacy of two types of diet with different macronutrients, on preventing or delaying the onset of type 2 diabetes in subjects with either impaired fasting glucose or impaired glucose tolerance. Three hundred and twenty-two subjects with prediabetes were randomly assigned to a high monounsaturated fat diet, normal fat diet, or control groups and followed for 2 years. For calculating the daily energy requirement in subjects with BMI ≥ 25 kg/m^2, the weight was adjusted with the use of 110% of the ideal body weight with no attempt for losing body weight. There was no difference in body weight decrease among the three groups after 2 years. The cumulative incidence of diabetes was 9.3% (95% CI, 3.6–14%) in the high monounsaturated fat diet (HMD) group, 13.2% (95% CI, 6.4–19%) in the normal fat diet (NFD) group, and 18.3% (95% CI, 10–25%) in the control group. The cumulative incidence of diabetes was 57% lower in the HMD group than that in the control group (95% CI, 0.1–0.9%; P = 0.03). This value was not significant in the NFD group (RR, 0.60; 95% CI, 0.2–1.2%; P = 0.1). Type 2 diabetes can be prevented by a high-monounsaturated fat, low-carbohydrate diet and receiving energy based on the adjusted ideal body weight without a weight loss program. Trial registration number: NCT02250066

Keywords Prediabetes · Impaired fasting glucose · Impaired glucose tolerance · Monounsaturated fatty acid · Olive oil

Abbreviations

HMD	High-monounsaturated fat diet
MUFA	Monounsaturated Fatty Acid
NFD	Normal fat diet
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
WHIDMT	Women's Health Initiative Dietary Modification Trial

Introduction

The prevalence of prediabetes has been steadily increasing. The goals of diabetes prevention are delaying the onset of diabetes [1, 2]. The individuals demonstrably at the highest risk for developing diabetes include those with impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), especially those with combined IFG and IGT. Subjects with additional diabetes risk factors, including obesity and family history, are more likely to develop diabetes. Changes in lifestyle, including diet modification, weight loss, and exercises, slow the progression of prediabetes to overt diabetes [3]. The benefit of exercise in preventing diabetes has been demonstrated in several studies [4, 5]. There is also evidence that lifestyle intervention (combined diet and exercise aimed at weight loss and increasing activity levels) can improve glucose tolerance and prevent progression from IGT to type 2

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diabetes [3]. There are few trials that examined the effects of diet alone on the prevention of diabetes [6]. Thus, our study was conducted to determine the effects of two types of diet, a high-monounsaturated fat diet (HMD) or a normal fat diet (NFD), without a weight loss program on preventing or delaying the onset of type 2 diabetes in subjects with either IFG or IGT.

Materials and methods

This study had two phases. The first phase of the study was cross sectional and was done by census on adults over 20 in a small city in 2012 [7]. Altogether, blood sampling was done from 3915 adults. Diagnosis of prediabetes was done by the latest American Diabetes Association criteria with a fasting glucose level of 100–125 mg/dL (5.6–6.9 mmol/L) or a 2-h post-glucose challenge in the range of 140–199 mg/dL (7.8–11.0 mmol/L), confirmed by two tests [8]. Three hundred and thirty-six participants with prediabetes were found. The second phase of the study was a parallel randomized controlled clinical trial. The study design was approved by the ethics committee in the university and informed consent was obtained from all participants. Exclusion criteria in the second phase were the ones on diet therapy, severe physical activity, accompanying diseases such as cancer which reduce their life expectancy, and the ones receiving thiazide diuretics, systemic ß blockers, glucocorticoids, nicotinic acid, and weight-lowering drugs. The participants were randomized and stratified into a control group and two study groups according to blood glucose (IFG and IGT or both) by block randomization. The block randomization was performed by a trained dietician.

Data collection

Baseline assessments were anthropometric measurements, food diary, and laboratory exams. Anthropometric measurements were done for all the participants by a dietitian to minimize the error of the measurements. The weight of the participants was measured while the subjects were minimally clothed and barefoot using digital scales and recorded to the nearest 0.1 kg. Height was measured in a standing position while barefoot using a tape meter while the shoulders were in a normal state. BMI was calculated as weight in kilograms divided by height in meters squared. Overweight and obesity were defined as $25 \leq \text{BMI} < 30$ kg/m^2 and BMI ≥ 30 kg/m^2, respectively. Waist circumference was obtained by measuring the distance around the smallest area below the rib cage and above the umbilicus with the use of a nonstretch tape measure, without any pressure to body surface, and recorded to the nearest 0.1 cm. Anthropometric measurements were repeated annually. Blood sample tests measured fasting blood glucose, glucose 2 h after ingestion of 75 g glucose, HDL cholesterol, LDL cholesterol, and triglyceride (TG). The same assessments were repeated annually (at 12 and 24 months) in all the groups. The investigators and the participants were unaware of the results and those who were diagnosed as having diabetes during the study were referred for treatment. Dietary intakes were calculated through a three-consecutive-day food diary of which 1 day was a holiday. It was done for each participant at baseline and at 6-month intervals by a dietitian. In the control group, dietary advice was given to subjects on each session. In the intervention groups, participants received detailed and individualized counseling. No information was given to change the level of physical activity. Information on physical activity was collected using the self-reported Modifiable Activity Questionnaire (MAQ) [9]. Total weekly leisure-time energy expenditure was obtained by summing the values for the individual activities. All smokers were also advised to stop smoking.

Interventions

Daily energy requirement was calculated for each subject in the intervention groups. For patients with BMI $\geq$25 kg/m^2, the weight was adjusted with use of 110% of the ideal body weight. Diet in the NFD group was 15% from protein, 30% from fat (10% MUFA, 10% PUFA, 10% saturated fatty acid (SFA)), and 55% from carbohydrate. Diet in the HMD group was 15% from protein, 45% from fat (25% MUFA, 10% PUFA, 10% SFA), and 40% from carbohydrate. The source of MUFA in this group was olive oil. In the intervention groups, a diet regimen was written for each participant by a dietitian. The control group was encouraged to follow the USDA Food Guide Pyramid and reduce their fat intake to less than 30% of energy consumption and saturated fat to less than 10% of total energy.

Statistical analysis

The primary outcome variable was incidence of type 2 diabetes mellitus, diagnosed by the latest American Diabetes Association criteria with fasting glucose level of $\geq$126 mg/dL (7.0 mmol/L) or a 2-h postglucose challenge $\geq$200 mg/dL (11.1 mmol/L) confirmed by two tests. SPSS software version 16 was used for data analysis. The minimum sample size estimated for each group was 66 at a power $(1 - \beta)$ of 90% and $\alpha = 0.05$ for a parallel interventional study with two-tailed testing to detect an incidence difference of 27% among groups for diabetes based on $P_1 = 68\%$ and $P_2 = 41\%$ obtained from the study by Pan XR et al. [10]. Changes in variables between groups were calculated by analysis of variance. The Cox regression analysis was used to estimate the relative risk (RR) of diabetes with 95% CI in the groups. We also conducted analyses, stratified according to the type of abnormal

glucose metabolism, sex, and body mass index by chi-square test. Paired *t* test was used for the within-group comparison between the baseline and the 2-year visit. $P \leq 0.05$ was considered statistically significant.

Results

Of the 336 patients enrolled in the study, three patients in the control group, five patients in the HMD group, and six patients in the NFD group were excluded because of refusing to continue the study, getting diet therapy, migration, or death (Fig. 1). Ultimately, 109 subjects in the control group, 107 in the HMD group, and 106 in the NFD group completed the study. The baseline characteristics of the subjects are shown in Table 1. No significant differences in baseline characteristics were observed among the groups.

Changes in diet

Baseline caloric intake and diet composition were similar in intervention groups. After 2 years of follow-up, estimated caloric intakes were lower in the intervention groups. Analysis of calorie composition showed a lower proportion of carbohydrates and a higher proportion of MUFA in the HMD group at follow-up, and the differences were statistically significant (Table 2).

Changes in anthropometric parameters

Proportions of BMI categories (<25 and ≥25) did not differ among the groups (Table 1). Table 3 shows the difference between anthropometric variables after 2 years. There was no significant difference in weight and waist circumference of the subjects in the HMD, NFD, and control groups at the end of the 24-month intervention.

Changes in lipid profile and blood glucose

Prevalence of IFG, IGT, and both abnormalities were similar within the three groups (Table 1).

Table 3 shows the difference between the metabolic parameters after 2 years. After 2 years, analysis of variance showed that fasting plasma glucose concentration and 2 h postprandial glucose concentration decreased more significantly among the subjects in the HMD and NFD groups compared with those in the control group. When comparing mean glucose levels before and after 2 years in each group, the only significant difference was observed in the HMD group in both fasting and 2 h postprandial plasma glucose concentrations.

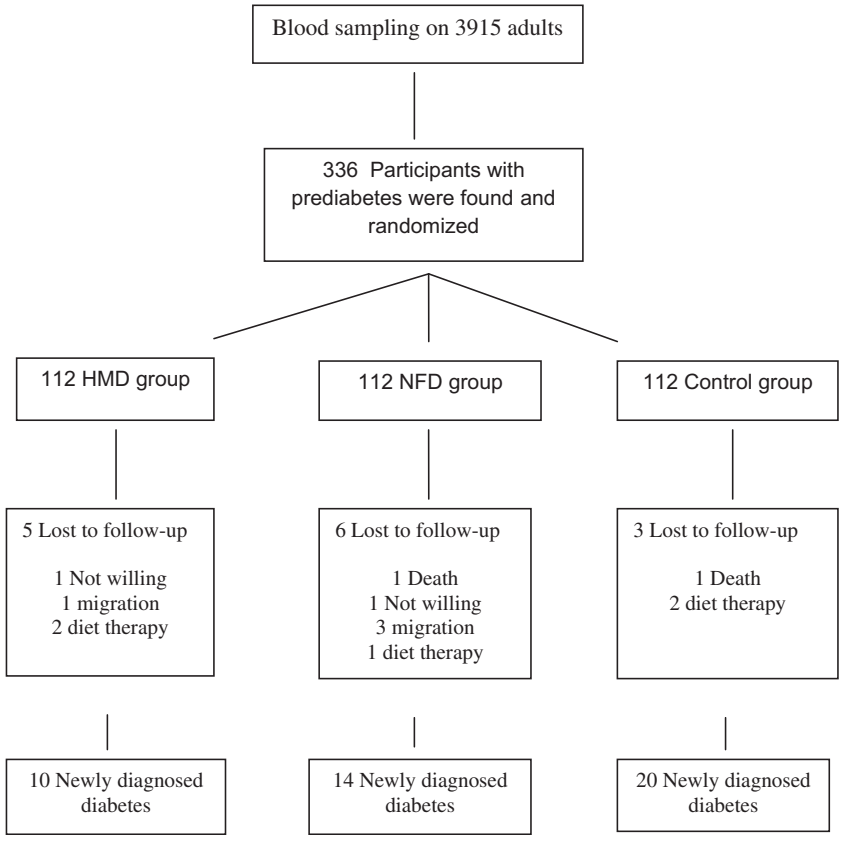

Fig. 1 Flowchart of the study participants

Table 1 Baseline characteristics of the subjects

	HMD (N = 107)	NFD (N = 106)	Control (N = 109)	P
Age	43.9 ± 10.2	43.5 ± 9.5	43.4 ± 9.6	0.9
Sex (no, %)				0.9
Male	37 (33)	37 (33)	38 (38)	
Female	70 (33.3)	69 (32.9)	71 (33.8)	
Smoking (no, %)	14 (32.6)	10 (23.3)	19 (44.2)	0.2
Alcohol (no, %)	11 (27.5)	12 (30)	17 (42.5)	0.4
BMI (kg/m^2) (no, %)				0.9
<25	43 (33.3)	43 (33.3)	43 (33.3)	
≥25	64 (33.2)	63 (32.6)	66 (34.2)	
WC (cm)	97.2 ± 17. 4	95.9 ± 16.9	96.8 ± 18.8	0.8
Wt (kg)	76.9 ± 15.7	78.5 ± 15.3	74.2 ± 13.8	0.1
Prediabetes type (no, %)				0.9
IGT	56 (32.9)	55 (32.4)	59 (34.7)	
IFG	36 (34)	36 (34)	34 (32.1)	
Both	15 (32.6)	15 (32.6)	16 (34.8)	
Leisure physical activity (MET/min/wk)	190 ± 109.1	207.3 ± 121.9	198.2 ± 103.3	0.5
Plasma lipids (mg/dL)				
LDL	142.1 ± 14.8	144.2 ± 15.4	141.1 ± 17.6	0.3
HDL	45.1 ± 5.1	45.1 ± 4.5	44.5 ± 5.1	0.5
Triglycerides	173.9 ± 33.8	174.8 ± 31.1	172.9 ± 37.9	0.9
Blood pressure (mm Hg)				
Systolic	127.3 ± 13.7	124.3 ± 13.9	125.1 ± 13.9	0.2
Diastolic	80 ± 8.9	79.8 ± 9.8	79 ± 8.6	0.6

HMD High monounsaturated diet, *NFD* normal fat diet, *BMI* body mass index, WC waist circumference, *W* weight, *MET/min/wk* metabolic equivalent

Table 2 Dietary intakes of subjects during the intervention period

	HMD	NFD	Control	P_2
Total calorie				
Baseline	2150 ± 659	2174 ± 590	2180 ± 615	0.7
Year 2	1985 ± 578[a]	2006 ± 615[a]	2192 ± 805[b]	0.04
P_1	0.04	0.05	0.7	
Carbohydrate (% intake)				
Baseline	56.2 ± 6.4	56 ± 5.1	56.5 ± 6	0.8
Year 2	43 ± 4.6[a]	55.6 ± 4.2[b]	55.8 ± 6.1[b]	0.001
P_1	0.03	0.3	0.4	
Protein (% intake)				
Baseline	12.5 ± 3.2	12.3 ± 2.8	12.4 ± 2.6	0.7
Year 2	14.7 ± 3.5	14.5 ± 3.1	14.2 ± 2.9	0.8
P_1	0.03	0.02	0.03	
Fat (% intake)				
Baseline	31.3 ± 4.7	31.7 ± 3.9	31.1 ± 4.5	0.9
Year 2	42.3 ± 3.9[a]	29.9 ± 3.8[b]	30 ± 5.7[b]	0.001
P_1	0.01	0.03	0.04	
MUFA[c] (%Fat)				
Baseline	6.8 ± 1	6.4 ± 1.4	6.7 ± 0.9	0.6
Year 2	23.3 ± 3.5[a]	9.5 ± 2.4[b]	8.9 ± 2[b]	0.001
P_1	0.001	0.04	0.04	

HMD High monounsaturated diet, *NFD* normal fat diet, *MUFA* monounsaturated fatty acid, P_1 with paired t test, P_2 with analysis of variance

[a, b, c] values with different superscript letters are significantly different

Table 3 Changes in anthropometric and metabolic parameters from the baseline to the end of 2 years

	HMD (N = 107)	NFD[b] (N = 106)	Control (N = 109)	P_2
Wt (kg)	−0.1 ± 0.7	−0.09 ± 0.6	0.2 ± 2.1	0.07
P_1	0.1	0.1	0.2	
WC (cm)	−0.6 ± 4.2[a]	−0.5 ± 3.8[a]	0.4 ± 3.7[b]	0.09
P_1	0.1	0.1	0.2	
Plasma glucose (mg/dL)				
Fasting state	−1.6 ± 8.2[a]	−1.4 ± 7.9[a]	4.3 ± 10.7[b]	0.001
P_1	0.04	0.06	0.001	
2Hpp	−3.9 ± 16.5[a]	−0.6 ± 17.7[a]	3.3 ± 14.8[b]	0.005
P_1	0.01	0.7	0.01	
Plasma lipids (mg/dL)				
LDL	−2.5 ± 7[a]	−2.9 ± 10.7[a]	1.4 ± 8.6[b]	0.001
P_1	0.005	0.001	0.09	
HDL	1.1 ± 3.3	1.0 ± 3	−0.06 ± 5.6	0.06
P_1	0.001	0.001	0.9	
Triglycerides	−12.8 ± 22.1[a]	−10.2 ± 21.7[a]	0.7 ± 17.5[b]	0.001
P_1	0.001	0.001	0.6	
Blood pressure (mm Hg)				
Systolic	−2.2 ± 13.6	−1.4 ± 11.8	−0.5 ± 9.8	0.5
P_1	0.09	0.2	0.5	
Diastolic	−0.4 ± 5.7	−0.5 ± 4.1	0.1 ± 3.5	0.4
P_1	0.3	0.2	0.5	

HMD High monounsaturated diet, *NFD* normal fat diet, *Wt* weight, *WC* waist circumference, *2Hpp* 2 h after an oral glucose load, P_1 with paired t test, P_2 with analysis of variance

[a, b, c] values with different superscript letters are significantly different

There was a significant decrease in serum triglycerides and LDL cholesterol in the HMD and NFD groups compared with those in the control group but the increase in HDL cholesterol level was not significant.

Incidence of diabetes

During this 24-month study, 44 participants (13.7%) were diagnosed as having type 2 diabetes: 10 in the HMD group, 14 in the NFD group, and 20 in the control group. After this period, the cumulative incidence of diabetes was 9.3% (95% CI, 3.6–14) in the HMD group, 13.2% (95% CI, 6.4–19) in the NFD group, and 18.3% (95% CI, 10–25) in the control group. According to the Cox regression analysis (adjusted for BMI, sex, age, cigarette, alcohol), the cumulative incidence of diabetes was 57% lower in the HMD group than that in the control group (95% CI, 0.1–0.9; P = 0.03). This value was not significant in the NFD group (RR, 0.60; 95% CI, 0.2–1.2; P = 0.1).

Cumulative incidence of type 2 diabetes based on sex in the HMD group was 13.5% (5) in males versus 7.1% (5) in

females ($P = 0.2$). These values in the NFD group were 16.2% (6) in males versus 11.6% (8) in females ($P = 0.5$) and for the control group, 18.4% (7) in males versus 18.3% (13) in females ($P = 0.9$).

Cumulative incidence of type 2 diabetes based on BMI <25 and BMI ≥25 was 0% (0) versus 15.6% (10) in the HMD group ($P = 0.006$). These values for the NFD group were 2.3% (1) versus 20.6% (13) ($P = 0.006$) and for the control group was 11.6% (5) versus 22.7% (15) ($P = 0.1$).

Cumulative incidence of type 2 diabetes by IFG, IGT, and both abnormalities were 5.6% (2), 7.1% (4), and 26.7% (4) in the HMD group ($P = 0.04$). In the NFD group, these values were 8.3% (3), 10.9% (6), and 33.3% (5), respectively ($P = 0.04$), and were 23.5% (8), 11.9% (7), and 31.2% (5), respectively, in the control group ($P = 0.1$).

Discussion

Our results showed that type 2 diabetes can be prevented or delayed in persons at high risk for this disease with a high-monounsaturated fat diet. The incidence of diabetes was reduced by 57% in this group. The benefit of exercise, weight reduction, and lifestyle change in preventing diabetes has been demonstrated in several studies [10–14]. There are few trials that examine the effects of diet alone on the prevention of diabetes. The effect of diet alone has been shown in the Women's Health Initiative Dietary Modification Trial. In this trial, over 48,000 postmenopausal women, not specifically at high risk for developing diabetes, were randomly assigned to a low-fat diet or to a usual diet. Weight loss and exercise were not part of the intervention. After an eight-year follow-up, there was no difference in the self-reported incidence of diabetes. These results suggest that in average-risk women, macronutrient change without weight reduction does not prevent diabetes [6]. A difference in our study with Women's Health Initiative Dietary Modification Trial (WHIDMT) was that we calculated the energy needs of all participants in the intervention groups and explained for them. This energy was calculated by adjusted body weight in overweight and obese subjects which did not lead to weight loss. Thus, all of the subjects received their energy intake based on their metabolically active tissues and no more than their body requirements. Another difference was in glucose testing which in WHIDMT there was no uniform glucose testing in participants. It seems that the type of macronutrient may be an important factor in preventing diabetes. In vitro and animal studies support the substitution of MUFA for saturated fat in the diet of subjects with diabetes because it has positive effects on glucose metabolism. Islet cells of humans show ß cell apoptosis, decreased ß cell proliferation, and impaired ß cell function by the saturated palmitic acid and elevated glucose concentration. These effects can be prevented by monounsaturated fatty acids [15–18]. There is no study to assay the effects of nonMediterranean high MUFAs on blood glucose and lipid profile of subjects with prediabetes but several studies on type 2 diabetes have shown that high-MUFA diets might have metabolic benefits.

In a meta-analysis of randomized, crossover trials in adults with diabetes, high-MUFA intake improved the blood glucose, triglyceride, and total and HDL cholesterol concentrations, but not A1c or LDL cholesterol levels [19]. In a clinical trial of individuals with metabolic syndrome, a greater improvement in body weight and cardiovascular risk factors (e.g., blood pressure, total cholesterol, HDL cholesterol, triglycerides, insulin resistance, and inflammatory markers) was seen in a Mediterranean diet group compared with those in a low-fat diet group [20]. In a one-year comparison in 124 patients with type 2 diabetes, both groups had similar weight loss and comparable improvement in body fat, waist circumference, diastolic blood pressure, HDL cholesterol, A1C, and fasting glucose and insulin levels [21]. The effects of high-MUFA and high-carbohydrate (CHO) diets on body weight and glycemic control were similar, too. In this study, subjects with a BMI of 27–40 kg/m^2 were selected for intervention. The same effect on blood glucose and lipid parameters which were seen in two groups may be due to different mechanisms of weight loss. In a six-month follow-up of adults with type 2 diabetes, insulin doses were reduced significantly more in the high-fat low-carbohydrate diet in comparison with those in the low-fat high-carbohydrate diet [22].

In our study, the effect of both intervention groups on the prevention of diabetes was greater in subjects with BMI < 25 than that in overweight and obese ones. Thus, it seems that macronutrient changes are more effective in subjects with a normal BMI. Also, a balanced calorie intake based on body needs is another important factor in preventing diabetes in subjects with BMI < 25.

These points may be the reason for a significant decrease in serum triglycerides and LDL cholesterol in the HMD and NFD groups compared with that in the control group. Our study is the first long-term randomized controlled trial to compare the effects of a high-MUFA diet and normal fat diet on the incidence of diabetes without a weight loss program. There are several important study limitations. First, the sample size in this trial was inadequate for measuring the incidence of diabetes because we found only 336 subjects with prediabetes by the census. The decrease in the incidence of diabetes in the NFD group might be significant if we had an adequate sample size. Also, our sample size was inadequate to assess the significance of the effects within the subgroups. Furthermore, we performed a food dairy analysis for each participant at 6-month intervals. Shorter intervals were preferred to assess the compliance level of participants to the diet.

Conclusion

High-monounsaturated fat low-carbohydrate diet, and calorie intake based on body needs may slow the progression of impaired glucose tolerance to overt diabetes. These effects are more prominent in subjects with a normal BMI. It seems that a combination of a weight loss program and changes in macronutrient composition in overweight and obese subjects is a better strategy which deserves further larger studies.

Acknowledgments We convey our gratitude to the Ilam University of Medical Sciences, Ilam, Iran. The contributions of the authors to the present study were as follows: Zahra Vahdat Shariatpanahi for the design of the experiment and analysis and interpretation of data; Shaahin Shahbazi for the design of the experiment and writing of the manuscript.

Compliance with ethical standards Funding of this study was provided by the Ilam University of Medical Sciences. The manuscript has been read and approved by all the authors. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):75–79
DOI 10.1007/s13410-017-0559-0

ORIGINAL ARTICLE

Serum oxidized LDL and the factors associated with LDL oxidation in black South African type 2 diabetes mellitus patients

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Received: 15 November 2016 / Accepted: 8 May 2017 / Published online: 7 June 2017

Abstract Diabetes is a metabolic disorder that is increasing in prevalence at a very fast pace in developing countries such as South Africa. There is a strong link between diabetes and cardiovascular disease with studies showing that diabetic patients are two to four times more likely to suffer from cardiovascular disease than healthy people. The dysregulation of lipid and lipoprotein metabolism and the subsequent oxidation of low-density lipoprotein (LDL) is one of the major reasons for the increased cardiovascular risk associated with diabetes. The main objective of this study was to analyze the concentrations of oxidized LDL in diabetic patients and to uncover factors associated with oxidized LDL. In total, 67 type 2 diabetic mellitus patients and 48 healthy control participants participated in the cross-sectional study. Oxidized LDL concentration in serum and the total antioxidant status were determined by ELISA, while the size of LDL and high-density lipoprotein (HDL) particles were determined by gradient gel electrophoresis. The concentration of oxidized LDL was significantly elevated in the diabetic patients. The diabetic patients also had a significantly higher percentage of small dense LDL and a higher concentration of triglycerides than the control group, while their peak LDL size and the total antioxidant capacity were significantly lower than those in the control group. The percentage of small dense LDL, triglycerides, peak LDL size, and total antioxidant capacity also varied significantly across the quartiles of oxidized LDL in the entire study population. Multiple regression analysis revealed that triglyceride and glycated hemoglobin were the most important parameters to be independently associated with oxidized LDL concentrations. The diabetic patients had high concentrations of small dense LDL and oxidized LDL. The concentrations of the highly atherogenic oxidized LDL particles were found to be independently associated with the concentrations of triglycerides and glycated hemoglobin. This indicated that proper diabetic control could reduce the risk of cardiovascular disease in these individuals.

Keywords Type 2 diabetes mellitus · Oxidized LDL · Peak LDL size · Triglycerides · Total antioxidant capacity

Introduction

Diabetes is a metabolic disorder characterized by chronic hyperglycemia. The incidence of diabetes is increasing at a very fast pace in developing countries due to urbanization and the resulting changes in lifestyle [1]. Peer et al., in a study on urban South Africans, reported that the prevalence of diabetes was 13.1% and that of impaired glucose tolerance (IGT) was 11.2% [2]. When compared to normal healthy individuals, diabetic patients have an accelerated rate of atherosclerosis that exposes them to more than twice the risk of mortality due to cardiovascular disease [3]. Oxidized LDL (ox-LDL) has been implicated in the progression of atherosclerosis and diabetic complications [4]. The chemical composition of LDL makes it susceptible to oxidation. Both the lipids and the apo B protein of LDL are subject to oxidative modification [5]. It is speculated that the oxidation of LDL occurs in the subendothelial space of the arteries due to the exposure of the LDL molecule to various cell-derived oxidants. Smaller LDL particles are more likely to penetrate the arterial wall and undergo oxidation [6]. Several factors such as size,

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concentration, and glycation of LDL particles have been implicated in the oxidation of LDL molecules. In addition, the free radical concentration and the scavenging capacity of the antioxidants in the serum can also affect the oxidation of the LDL molecule [7]. The oxidatively modified LDL molecule is recognized not by the LDL receptor but by scavenger receptors on the macrophages [8]. Internalization of this ox-LDL results in the activation of various intracellular pathways that can cause injury to the cell and lead to the formation of foam cells which play a key role in early atherogenesis [9].

Hence, this study was carried out to study the concentration of ox-LDL in non-diabetic and diabetic black South Africans and to access the factors associated with the oxidation of LDL.

Patients and methods

This study was cross-sectional in nature and was approved by the Health Research Ethics and Bio-Safety Committee of Walter Sisulu University. The analysis was carried out at the Medical Biochemistry Laboratory, WSU, Mthatha, and at National Health Laboratory Services (NHLS), Mthatha. Participants within the age group 35–65 years with a positive history of diabetes were included in the study. Participants with known diabetic complications, those on insulin treatment and lipid-lowering medication, were all excluded. Healthy control participants were recruited from the local population. A total of 140 participants were recruited for the study, following informed consent. Interviewer-administered questionnaires were used to obtain information on the duration of diabetes, medication, and lifestyle. Blood pressure and anthropometric measurements of all participants were also taken. Body mass index (BMI) was calculated as the weight (kg)/height $(m)^2$. Blood and urine samples were collected from the participants following an overnight fast into appropriate vacutainer tubes and sterile urine containers. The serum or plasma was separated within an hour and stored at −80 °C for analysis. A Roche Cobas 6000 autoanalyzer was used in the analysis of fasting blood sugar (FBS), glycated hemoglobin (HbA1c), lipid profile, liver function tests, urinary albumin, and creatinine. Following the analysis of results, participants with signs of infection and liver and kidney problems were excluded, and finally, 115 participants (48 controls and 67 patients with type 2 diabetes) were selected for the analysis. Of the 115 participants, 40 were male participants (15 control and 25 diabetic) and 75 were female (33 control and 42 diabetic). The serum total antioxidant capacity (TAO) was measured by a commercial kit from Sigma-Aldrich using the ABTS method. Ox-LDL was determined using a sandwich ELISA kit from Mercodia (Uppsala, Sweden). The absorbance was read using a Biotek analyzer at 450 nm. The size of LDL and HDL was analyzed using composite 2–31% gradient gels from Alamo Gels Inc., San Diego, USA. The electrophoresis was carried out on CBS electrophoresis units (USA), according to the protocol described by Singh et al. [10]. The images of the gels were analyzed using the gel analysis software, Gel Analyzer 2010. The fractional absorbance above 25.5 nm was summed up and classified as large LDL, while the absorbance between 21 and 25.5 nm were considered to be small dense LDL (sd-LDL). Similarly, HDL bands between 13 and 8.2 nm were considered to be large HDL, while those between 8.2 and 7.2 nm were considered to be small HDL [10]. The amounts of the small and large LDL and HDL molecules were expressed in percentages.

Statistical analysis

The data was analyzed statistically using the IBM statistical package for the Social Sciences (SPSS Inc., Chicago, IL, USA version 16). The Shapiro-Wilk test was used to check for normality of the data. Normally distributed data were expressed as mean ± standard deviation (SD) and the independent sample *t* test was done to check for differences between groups. Data which did not exhibit normal distribution were expressed as median and interquartile ranges, and the difference between groups was determined by the Mann-Whitney *U* test.

Pearson or Spearman correlation was performed to analyze correlations between the variables within groups. Multiple linear regression was used to further analyze the correlations between ox-LDL and other variables. A p value of <0.05 was considered as statistically significant.

Results

The mean age of the controls was 54.7 ± 6.1 years and that of the diabetic patients was 55.9 ± 5.5 years. The mean duration of diabetes was 7.5 ± 6.5 years. There was a high prevalence of obesity among both the controls and diabetic participants. Only 16% of the diabetic patients in this study had a normal BMI. The characteristics of the participants are given in Table 1.

Ox-LDL was significantly elevated in the diabetic group when compared to that in the control group ($p < 0.001$).

The peak LDL size ($p = 0.043$), TAO ($p = 0.001$), and HDL ($p = 0.008$) were significantly lowered in diabetics when compared to those in the control group. The triglyceride (TG) ($p < .001$) and sd-LDL percentage ($p < 0.001$) were significantly higher among the diabetics.

The concentration of ox-LDL correlated significantly and positively with the percentage of sd-LDL ($p = 0.047$, $r = 0.31$) and LDL ($p = 0.044$, $r = 0.34$), while it correlated negatively with HDL ($p = 0.003$, $r = -0.50$) and peak LDL size ($p = 0.048$, $r = -0.26$) in the control group.

In the diabetic group, ox-LDL correlated significantly and positively with total cholesterol (TC) ($p = 0.011$, $r = 0.34$),

Table 1 Clinical and metabolic characteristics of the control and diabetic participants

Variable	Control	Diabetic	*p* value
Age (years)	54.7 ± 6.1	55.9 ± 5.5	0.32
Diabetic duration (years)	NA	7.5 ± 6.5	
FBS (mmol/L)	4.9 (4.6–5.2)	8.3 (6.3–122.0)	<0.001*
HbA1c (%)	5.9 (5.7–6.1)	8.5 (6.9–10.0)	<0.001*
TC (mmol/L)	4.5 ± 0.88	4.8 ± 1.0	0.58
TG (mmol/L)	1.0 ± 0.34	1.4 ± 0.5	<0 .001*
HDL (mmol/L)	1.4 (1.1–1.6)	1.2 (1.0–1.4)	0.008*
LDL (mmol/L)	2.8 (2.2–3.2)	2.9 (2.2–3.4)	0.41
Ox-LDL (U/L)	73.9 ± 32.2	110 ± 42.1	<0.001*
BMI (kg/m^2)	28.6 (24.8–34.8)	30.1 (25.7–37.5)	0 .29
WHR	0.92 (0.87–0.95)	1.2 (1.1–1.2)	<0.001*
Peak LDL (nm)	26 (25.1–28.2)	25.4 (24.7–27.8)	0.043*
Sd-LDL (%)	28.5 (19.5–44)	38 (20.8–80.5)	0.003*
Peak HDL (nm)	10.8 (9.1–11.2)	10.69 (8.9–11.45)	0.47
HDL2 (%)	70 (58–80)	65 (49.5–79.5)	0.46
HDL3 (%)	29 (17.3–38.7)	34 (19–45.5)	0.24
TAO (mM)	0.6 (0.4–0.8)	0.4 (0.3–0.5)	0.001*

Data is significant at $p < 0.05$ level

LDL ($p = .004$, $r = 0.38$), sd-LDL percentage ($p = 0.014$, $r = 0.40$), and TG ($p = 0.013$, $r = 0.38$). The ox-LDL and HDL did not show a statistically significant correlation in the diabetic group. Ox-LDL correlated significantly and negatively with TAO ($p = 0.001$, $r = -0.45$) and peak LDL size ($p = 0.047$, $r = -0.29$).

The participants in the study were further divided into quartiles on the basis of their ox-LDL values; the results of which are summarized in Table 2. The FBS, HbA1c, waist-hip ratio (WHR), TC, LDL, TG, and sd-LDL percentage increased significantly across the quartiles of ox-LDL, while TAO decreased significantly as the ox-LDL increased. The peak LDL size also decreased as the ox-LDL increased; however, this was not statistically significant.

Multiple regression analysis was carried out to further analyze the association between variables like TAO, TG, and ox-LDL as shown in Table 3. Only TG and HbA1c were significantly associated with ox-LDL.

Discussion

This study was undertaken to understand the changes in concentration of ox-LDL as a consequence of the metabolic alterations in diabetic patients of African origin. There was no statistically significant difference in BMI between the diabetic and control groups. This could be due to the high prevalence of obesity in both the diabetic and control group. The waist-hip ratio, however, was significantly increased in the diabetic group. According to Vasquez et al., central obesity is more associated with disturbances in glucose homeostasis than with total adiposity [11]. Several other authors have found waist-hip ratio to be a good predictor of non-insulin dependent diabetes mellitus and cardiovascular disease [12–14]. The diabetic patients also had a statistically significant increase in the level of TC and TG and a decrease in HDL level. The increased TG and decreased HDL is a characteristic of diabetic dyslipidemia and might be due to abnormal insulin function in diabetics [15]. Insulin resistance results in an increase in both the flux of free fatty acids and the concentration of apo B protein which is produced by the liver [16, 17]. The main contributor to the higher apo B levels is the decreased degradation of apo B due

Table 2 Clinical and metabolic characteristics of participants across quartiles of ox-LDL

Variable	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*p* value
Age (years)	50.7 ± 11.4	53.3 ± 10.7	55.6 ± 7.1	56.9 ± 6.4	0 .2
FBS (mmol/L)	6.2 ± 2.7	6.6 ± 4.3	8.0 ± 4.3	9.5 ± 4.2	0.006*
HbA1c (%)	6.6 ± 1.9	7.1 ± 2.6	8.3 ± 2.6	8.5 ± 2.1	<0.001*
BMI (kg/m^2)	31 ± 7.2	28.4 ± 6.5	30.1 ± 6.6	34.9 ± 7.5	0.022*
WHR	0.89 ± .05	0.95 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.005*
TC (mmol/L)	4.2 ± 0.9	4.5 ± 0.9	4.7 ± 0.9	5.2 ± 1.0	0.006*
TG (mmol/L)	1.1 ± 0.3	1.2 ± 0.4	1.3 ± 0.5	1.5 ± 0.4	0.002*
LDL (mmol/L)	2.2 ± 0.8	2.6 ± 0.8	2.8 ± 0.8	3.2 ± 0.9	0.032*
HDL (mmol/L)	1.4 ± 0.3	1.3 ± 0.4	1.2 ± 0.6	1.3 ± 0.4	0.073
Peak LDL size (nm)	26.6 ± 0.7	26.2 ± 0.7	26.0 ± 0.6	25.2 ± 1.6	0.57
Sd-LDL (%)	30.3 ± 20.4	31.8 ± 25.1	44.1 ± 26.8	60.3 ± 25.1	0.027*
Peak HDL size (nm)	10.1 ± 1.1	10.6 ± 1.3	10.7 ± 1.4	11.3 ± 3.6	0.51
HDL2 (%)	66.9 ± 13.7	67.8 ± 15.5	66.7 ± 18.1	61.7 ± 1.9	0.8
HDL3 (%)	32.3 ± 13.3	27.7 ± 13.6	27.6 ± 16.2	36.1 ± 19.5	0.3
TAO (mM)	0.59 ± 0.19	0.46 ± 0.15	0.44 ± 0.27	0.33 ± 0.16	0.009*

Data is significant at $p < 0.05$ level

Table 3 Multiple regression analyses between ox-LDL and other variables

	B	Std. Error	Beta	*t*	Sig.
(Constant)	15.909	40.977		0.388	0.700
TAO	−21.770	21.720	−0.133	−1.002	0.322
Sd-LDL (%)	0.257	0.150	0.219	1.715	0.094
BMI	0.406	0.600	0.080	0.677	0.502
HbA1c (%)	6.158	2.961	0.300	2.080	0.044
TC (mmol/L)	−11.158	10.784	−0.269	−1.035	0.307
TG (mmol/L)	30.991	12.474	0.338	2.484	0.017
LDL (mmol/L)	14.853	12.348	0.312	1.203	0.236

to hepatic insulin resistance [18]. This results in an increase in concentration of the apo B-containing lipoproteins, mainly very low-density lipoprotein (VLDL) which is one of the major transporters of TG [19]. The increased VLDL concentration results in an increased exchange of TGs from VLDL to HDL and cholesterol from HDL to the VLDL particles. This exchange is mediated by the cholesterol ester transfer protein (CETP) [20]. The resulting increase in surface lipids in the HDL particles due to transfer from the TG-rich lipoproteins can result in defective lipolysis and a decreased concentration of HDL particles [20, 21]. There was little difference in the LDL levels between normal and diabetic individuals. This could be because the change in the LDL concentration in diabetes is more qualitative than quantitative. The most important qualitative modification is the oxidation of the LDL particle which is due to both the decreased size of the LDL particle and the increased level of oxidative stress [22]. The increase in sd-LDL concentration is accompanied by the decrease in concentration of large LDL fraction which results in hardly any change in the total LDL concentration [23].

The decreased size of LDL in diabetics is mainly because of the increased VLDL concentration which activates CETP. The activation of CETP results in an exchange of TG from the VLDL particle for cholesterol esters from the LDL particle. The TG-enriched LDL particle shows a higher affinity for hepatic lipase (HL) which results in hydrolysis of the triglycerides, and as a result, the LDL particles shrink and become more dense [24].

The study showed that the ox-LDL levels in diabetics were significantly elevated when compared to the normal healthy controls. This increase in ox-LDL concentration in diabetic patients has been observed in other studies [25]. The ox-LDL concentration was negatively correlated with the peak LDL size and positively with the sd-LDL percentage. The sd-LDL percentage also increased significantly with the increase in ox-LDL quartiles. The small LDL particles are highly atherogenic, as they can penetrate the arterial wall and enter the subendothelial space more easily, resulting in oxidation [6]. Furthermore, the triglyceride-enriched LDL particles, when compared to normal LDL particles, have a lower affinity to the LDL receptor and are retained longer in the plasma increasing its chances of being oxidized [26].

The increased oxidation of LDL in diabetes may also be due to increased glycation. In this study, the FBS and HbA1c increased significantly across the quartiles of ox-LDL. Chronic hyperglycemia in diabetic individuals can result in non-enzymatic glycation of the apo B protein. Glycated LDL molecules have a higher susceptibility to oxidation [27]. Hence, strict diabetic control can decrease the level of ox-LDL and can help reduce the risk of cardiovascular disease. Another factor that could be a cause of increased oxidation of LDL in diabetic patients is the decrease in TAO as observed in this study. Decreased TAO in diabetic patients has also been observed in several other studies [28, 29]. The oxidation of the LDL can be prevented by HDL-associated enzymes such as paraoxonase (PON-1) [30]. This could explain the significant correlation between ox-LDL and HDL in the control population. However, the ox-LDL concentration showed no significant correlation with HDL in the diabetic population. The lack of correlation between ox-LDL and HDL could indicate some dysfunction in the HDL particles. Hyperglycemia causes the glycation of HDL-associated proteins which results in HDL dysfunction in diabetics [31]. The activity of PON-1 has been observed to be negatively correlated with FBG in diabetic patients with poor glycemic control [32].

Hence, in this study, there was an increase in the atherogenic ox-LDL particles, among the diabetic individuals. The oxidation of the LDL particles was mainly dependent on the concentration of TG and the glycation of proteins as shown by the association between HbA1c and ox-LDL.

Conclusion

Diabetic patients have a high concentration of the highly atherogenic ox-LDL. The high concentration of ox-LDL among diabetics could be due to several factors such as an increased sd-LDL concentration, TG and HbA1c, along with a decrease in TAO. Since HbA1c level was independently associated with ox-LDL, proper diabetic control could decrease the risk of cardiovascular disease in these individuals. Furthermore, testing for sd-LDL and ox-LDL might give a better picture of dyslipidemia in diabetic individuals.

Limitations of the study

The incidence of cardiovascular disease among the participants was not assessed clinically.

The small sample size might affect the statistical analysis of the results.

Acknowledgements The authors would like to thank all the research participants who took part in this study, and Walter Sisulu University, South Africa, for funding this study.

Authors' contributions Jim Joseph: Study design; data acquisition; data analysis; statistical analysis; manuscript preparation
Farzana Ganjifrockwala: Data acquisition; manuscript editing
Grace George: Study design; statistical analysis; manuscript editing

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study conformed to the 1964 Helsinki declaration and its later amendments. The study was approved by the Health Research Ethics and Bio-Safety Committee of Walter Sisulu University (Protocol No: 013/012).

Statement of informed consent Written informed consent was obtained from all the participants included in the study.

Funding This study was funded by the research department of Walter Sisulu University, South Africa.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):80–87
DOI 10.1007/s13410-017-0561-6

ORIGINAL ARTICLE

Automatic detection of retinal hemorrhages by exploiting image processing techniques for screening retinal diseases in diabetic patients

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Received: 19 December 2016 / Accepted: 10 May 2017 / Published online: 2 June 2017

Abstract Diabetic retinopathy (DR) is one of the main retinal abnormalities which is asymptomatic and is the main cause of vision loss in diabetic patients. The computer-aided diagnosis systems based on image processing not only facilitate the doctor but also decrease the diagnosis time. This work represents the automated detection of one of the red lesion, i.e., hemorrhages, which are one of the most distinctive signs of retinal diseases in diabetic patients. In the proposed method, the foremost step is to enhance the image quality by eliminating the background noise and nonuniform illumination. This is achieved by applying the methods such as image contrast enhancement and normalization. The subsequent step is to segment the blood vessels from hemorrhages (using scale-based method) as both of them have the same color. The last step is to delineate the hemorrhages by exploiting the gamma correction and global thresholding techniques. The proposed method has achieved specificity (SP) of 84%, sensitivity (SN) of 87%, and an accuracy of 89% on the DIARETDB1 database.

Keywords Diabetic retinopathy · Hemorrhages · Red lesions · Exudates · Image normalization · Green channel

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Introduction

Diabetes is one of the major diseases being faced by the world today. World Health Organization(WHO) survey estimated that 2.8% people suffered from diabetes in 2000, and this percentage would increase to 4.4% in 2030 [1]. Diabetes is becoming common nowadays in people due to physical inactivity, obesity, and aging population. Diabetic retinopathy (DR) is a secondary microvascular complication of both type 1 and type 2 diabetes, the prevalence of which strongly correlates to both the duration of diabetes and the level of glycemic control as evidenced by diabetes control and complication trial (DCCT) and UK prospective diabetes study [2, 3].

DR is the most frequent cause of new cases of blindness among the adults aged 20–64 years in the developed countries [4]. It is classified into non-proliferative DR (NPDR) and proliferative DR (PDR) stages. The earliest clinical sign of NPDR includes microaneurysms which appear as small red dots in the superficial retinal layers and cause the retinal hemorrhaging. The dot and blot hemorrhages occur as microaneurysms rupture in the deeper layers of the retina such as the inner nuclear and the outer plexiform layer. This is followed by the flame-shaped hemorrhages which occur in more superficial layers of the retina. Later as the disease progresses, the cotton-wool spots, venous beading, and the intra retinal microvascular abnormalities develop, which are the hallmarks of the progressive capillary perfusion [5]. Neovascularization on the surface of the retina and the optic disc in conjunction with further retinal ischemia signifies the presence of the PDR [4, 6].

According to a study [7], after 20 years of diabetes, the chance of having DR in patients of type 1 diabetes increases by 99% and 60% in patients of type 2 diabetes. Owing to these alarming statistics, diabetic patients need to have regular screening of their eyes to prevent themselves from vision loss. Diabetic patients with limited access to ophthalmologists have low screening rates for DR [8]. As the prevalence of diabetes is projected to increase from 25 million to 125 million in the USA by 2050 [9], the number of patients with diabetes requiring the annual retinal evaluations will far exceed the capacity of the ophthalmologists. Recent studies have shown that the undiagnosed diabetes and the related retinopathy due to virtually non-existent primary eye care centers are common in the general population and are associated with the impaired visual status of the community. This is especially the case in the third world countries like Pakistan, where the resources are limited and the budgetary allocation to health is inadequate; hence, the prevalence of DR is increasing [3, 10]. The current detection process of DR is manual, costly, and time-consuming and requires specialized skills to operate the equipment. Therefore, a new paradigm for the care of these patients is needed. Periodic sequential retinal photos are rapidly becoming the standard of care for monitoring various ocular conditions such as glaucoma, diabetic and hypertensive retinopathy, and macular degeneration. The computer-aided diagnosis systems based on image processing are becoming common these days to facilitate the doctor and reduce the diagnostic time. Towards such ends, this work introduces the readers with yet another method to automatically detect one of the pathological signs of DR in diabetic patients earlier in the disease process, i.e., hemorrhages which appear after development of microaneurysms. The proposed method is based on the image processing techniques which involve three stages, i.e., preprocessing for image normalization and contrast adjustment, blood vessel segmentation, and lastly, the localization of hemorrhages by mainly exploiting gamma correction and global thresholding. Introducing this approach from the point of view of primary care physician would substantially reduce barriers and improve early screening of retinopathy in diabetic patients by detecting hemorrhages. Also, this approach in near future will prove to be cost-effective in DR screening of diabetic patients compared to conventional retinal examination.

Related work

The supervised learning methods require some prior labeling of information in order to classify pixel as a vessel pixel or non-vessel pixel. The rule for vessel segmentation is learned on the basis of training dataset by the algorithm. In the training set, vessels are precisely segmented and marked manually by expert ophthalmologist in order to provide ground truth for learning process of the algorithm. The supervised methods are based on preclassified data; hence, their performances are better than those of the unsupervised approach.

The blood vessels in retinal images appear darker than their surrounding. This characteristic of the blood vessel was exploited by Marin et al. [11]. They proposed five gray-level and seven moment invariant (known as Hu moment invariant based) feature descriptors in combination with multilayered feed forward neural networks as a classifier that has 7 neurons in the input layer and 15 neurons in three hidden layers while output layer consists of one neuron only. The proposed algorithm proved to be robust and effective on multiple-image database and with different image variations. The AUC, accuracy, specificity, and sensitivity of the proposed methods on STARE database are 0.9769, 0.9526, 98.19%, and 69.44%, respectively, and 0.9588, 0.9452, 98.01%, and 70.67%, respectively, on DRIVE.

Similarly, Shanmugam and Banu [12] proposed five gray-level-based and two moment invariant-based features in combination with extreme learning machine (ELM) classifier for vessel segmentation in the retinal image. The proposed technique has 0.9862 accuracy, 96.79% specificity, and 82.74% sensitivity on STARE database while the same algorithm has 0.9725 accuracy, 96.79% specificity, and 81.94% sensitivity on DRIVE database. Preethi and Vanithamani [13], proposed the use of moment invariant features with neural network and morphological processing in combination with support vector machine (SVM) as classifier and scored the accuracy of 0.9365 and 0.955, respectively. Akita and Kuga [14] and Nekovei and Sun [15] exploited the artificial neural network and back propagation neural network respectively for blood vessel segmentation. However, the results of both methods were produced by visual inspection.

Sinthanayothin et al. [16] proposed a segmentation technique in which principle component analysis (PCA) was used in combination with neural network for vessel segmentation and achieved the specificity of 83.3% and sensitivity of 91%. Niemeijer et al. [17] utilized gaussian matched filter, and first- and second-order gaussian derivatives on different scales with k-nearest neighbor (k-NN) classifier for vessel segmentation, and got the accuracy of 0.9416. Staal et al. [18] exploited the intrinsic property of retinal blood vessels, found that the vessels are elongated structure, proposed image ridge-based blood vessel segmentation, and used k-NN classifier for classification purposes. The proposed image processing algorithm achieved 0.9614 of area under the curve of ROC and 0.9516 accuracy on publicly available STARE database.

You et al. [19] employed a combination of radial projection and support vector machine (SVM) classifier for

retinal blood vessel segmentation. The proposed method is effective in the detection of low contrast and narrow blood vessels. The accuracy, sensitivity, and specificity of proposed technique on STARE database are 0.9497, 72.6%, and 97.56%, and for DRIVE, they are 0.9434, 74.1%, and 97.51%, respectively. The line operator with SVM classifier for vessel segmentation was proposed by Ricci et al. [20]. The proposed method is robust to nonuniform illumination and computationally simple, and requires few features. The method has the area under ROC curve as 0.9558 and 0.9602 and average accuracy as 0.9563 and 0.9584 for DRIVE and STARE database, respectively. Soares et al. [21] used 2D Gabor wavelet at multiple scales with Gaussian mixer model (GMM) classifier for segmentation of blood vessels. The proposed method has limitation with images containing nonuniform illumination and did false detection in such cases. The accuracy and AUC of this method are 0.9466 and 0.9614 on DRIVE and 0.9480 and 0.9671 on STARE database, respectively. Similarly, Osareh and Shadgar [22] utilized Gabor filter at multiple scales for candidate identification and PCA as feature extractor in combination with SVM and GMM as classifiers. The accuracy, sensitivity, and specificity of the method are 0.9675, 96.5%, and 97.10% with SVM as classifier, respectively, and 0.9524, 96.14%, and 94.84% with GMM as classifier, respectively. Lupascu et al. [23] employed 41 D feature vector containing the output of various filters (Gaussian, derivative of Gaussian, 2D Gabor filter, and matched filter) in combination with AdaBoost classifier and scored the accuracy of 0.9597, 98.74% specificity, and 67.28% sensitivity. Tamilarasi et al. [24] proposed Genetic based Fuzzy Seeded Region Growing segmentation for detection of exudate in fundus images. The proposed method achieved accuracy, specificity, and sensitivity of 0.9938, 98%, and 81.55%, respectively.

The detection of hemorrhages in retinal images [25] was also investigated by using morphological methods followed by analog algorithms. The proposed methodology was tested on many of the images, and average sensitivity, specificity, and probability values came out to be 81.9, 99.9, and 92.0%, respectively.

Shivaram et al. [26] used mathematical morphological operations and image arithmetic methods for the detection of hemorrhages and to repress the blood vessels. The result was validated by ophthalmologist and it was also compared with the hand drawn ground-truth images. After comparison, their sensitivity, specificity, and predicted value are computed as 89.49, 99.89, and 98.34%, respectively.

Sinthanayothin et al. [27] detected some symptoms of non-proliferative diabetic retinopathy using the recursive region growing segmentation, and the proposed methodology has sensitivity of 77.5% and specificity of 88.7%. These results were produced after the direct comparison of the resultant images with the image on which symptom identifications and markings have been done by the ophthalmologist.

Adaptive thresholding technique was used by Devaraj [28] to detect red lesions. His proposed method adjusted the image contrast and morphological operation erosion was applied to achieve better results. The methodology was tested on images from DIARETDBI database.

Matei and Matei [29], morphological operations using the cellular neural network operators are proposed to detect the hemorrhages in the retinal image followed by the erosion to remove blood vessels. Barman et al. [30] combined neural network with the tracking algorithm to spot the hemorrhages in fundus image. This technique was used to handle the misclassification where small hemorrhages were classified as vessels. The tracking algorithm helps in differentiating between the vessels and hemorrhages.

Diagnosis of diabetes by the detection of hemorrhages, microaneurysms, and exudates was done by using morphological image processing techniques along with SVM. These techniques were proposed by Acharya et al. [31] having sensitivity of 82% and specificity to be 86%.

Jagatheesh and Jenila [32], DR lesions are detected using bag of visual words (BoVW) model approach. The required features were extracted using speeded up robust features (SURF). For building visual dictionary, k-means clustering was used. For generating bag of visual words (BOVW), Fisher vector encoding and max pooling technique are applied followed by SVM used for lesion classification.

Kleawsirikul et al. [33], an attempt has been made to develop an effective algorithm to detect hemorrhages. In the preprocessing step, the green channel was extracted from the fundus image as it distinctly displays the red color features of both hemorrhages and retinal blood vessels. The image is then inverted in order to emphasize the areas of interest, i.e., the hemorrhages, in white, and then, contrast-limited adaptive histogram equalization (CLAHE) is applied to maximize the contrast of the image. Following this, the morphological top hat operator was applied to detect the hemorrhages. Finally, rule-based classification is used to classify hemorrhages based on their features, for instance, compactness, area, and eccentricity. The proposed method achieved the sensitivity of 80.37%, specificity of 99.53%, and accuracy of 99.12%.

Seoud et al. [34], used dynamic shape feature to identify the red lesions in the fundus image. The proposed method does not require prior segmentation of the blood vessels, and the candidate regions are identified after image processing and features are then extracted to subsequently classify each potential region. For classification, supervised learning was used to differentiate the lesions from other structures and background noise. The method was tested on several databases; few of which are publicly accessible also. When

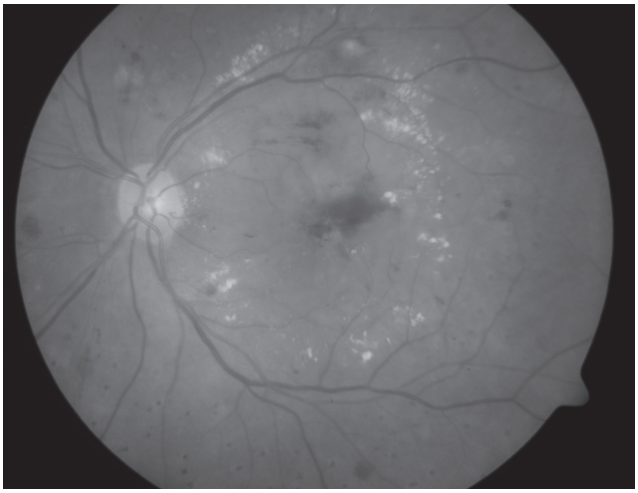


Fig. 1 Input image

tested on the Retinopathy Online Challenge's database, this method obtained a free-response receiver operating characteristic (FROC) score of 0.420 which ranked it fourth. On the Messidor database, this method achieved an area under the ROC curve of 0.899.

The work presented in this paper is also inspired by the computerized detection of hemorrhages which is one of the early signs of the presence of the retinal diseases in the diabetic patients after the development of microaneurysms.

Implementation

The approach presented in this paper for detecting hemorrhages is based on three major steps. The first step is to preprocess and enhance the image quality in case of low illumination and contrast. The subsequent step is to remove blood vessels from the fundus image. This is a good starting point which will assist in not detecting blood vessels as hemorrhages. Both blood vessels and hemorrhages share the same color so it is important to detect the blood vessel first and then segment it from the fundus image. After the blood vessel segmentation, the later steps mostly employ the morphological operations and thresholding. All steps will be performed on the input image as shown in Fig. 1.

Preprocessing

There might be cases when the fundus image suffers from poor illumination and contrast. In such cases, before detecting the hemorrhages, the preprocessing of the fundus image becomes indispensable. The purpose of preprocessing is to enhance the image contrast and its brightness. To increase the contrast of the fundus image, we first extracted the green channel response of the image as it provides a better contrast as compared to the other two color channels, i.e., blue and red. Although we increased the contrast of the image, the contrast alone is not sufficient to detect the hemorrhages. The green channel response of the image was further normalized using Eq. 1:

$$n(x, y) = (g(x, y) - G_1) \div \sqrt{G_2}, \quad (1)$$

where $n(x, y)$ is the normalized image, $g(x, y)$ is the input image, and G_1 and G_2 are the Gaussian filters. Equation 1 can be comprehended more easily by Fig. 2.

G_1 is the Gaussian filter of $g(x, y)$ with sigma σ_1, and size of the filter is the double of normal inverse cumulative distribution function of σ_1 with mean 0 and probability $1e - 1$.

G_2 is the Gaussian filter of square of $g(x, y)$ with sigma σ_2 and size the double of normal inverse cumulative distribution function of σ_2 with mean 0 and probability $1e - 1$. Figure 3 shows the preprocessing result.

Blood vessel segmentation

After preprocessing, the blood vessel segmentation is performed. It is a prerequisite in detection of hemorrhages and a complex problem. We used the method proposed by Vlachos and Dermatas [35]), in which multi-scale retinal vessel segmentation has been performed using line-tracking. The line-tracking process begins from a small cluster of pixels, acquired from a brightness selection rule, and aborts when a cross-sectional profile condition is eventually invalid. The multi-scale image projection is obtained after combining each image map along scales, encompassing the pixels confidence to exist in a vessel. The foremost network of vessels is obtained after performing map quantization of the multi-scale confidence matrix. Then, median

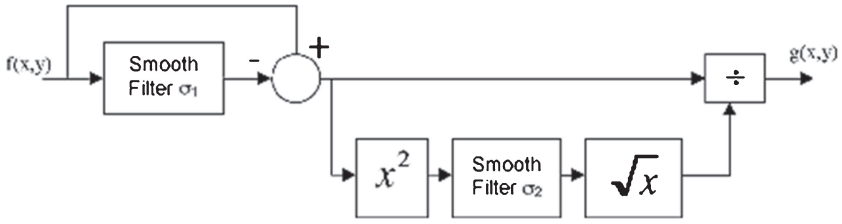
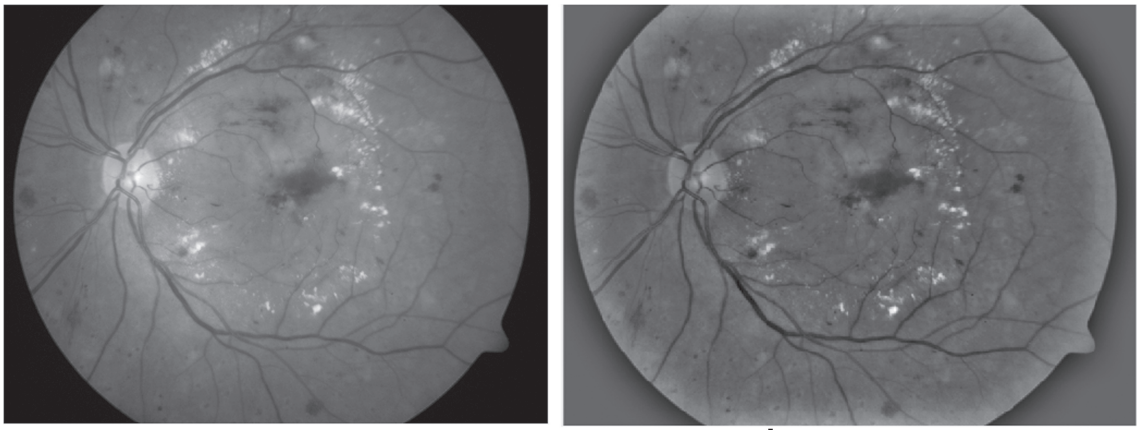


Fig. 2 Image normalization



a Green channel response **b** Normalized image

Fig. 3 Preprocessing

filtering is adapted in the foremost vessel network, rebuilding disjointed vessel lines and removing the noisy lines. Eventually, postprocessing eliminates the erroneous areas by applying the directional attributes of the vessels and the morphological reconstruction (Fig. 4a).

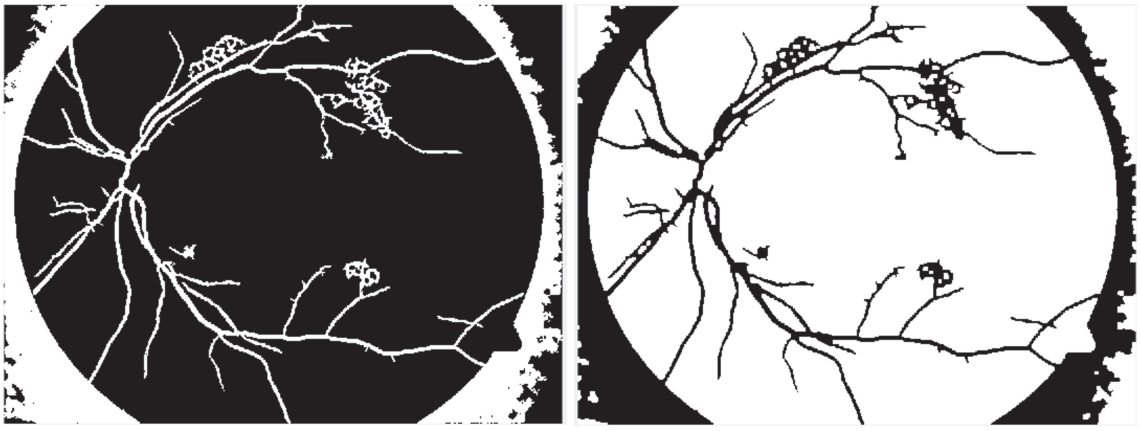
The image obtained after blood vessel segmentation is further processed using morphological opening operation with square structuring element. With morphological opening, the entire foreground morphology which is smaller than the structuring element is removed by applying erosion, and then, the residual structures are softened or smoothed by using dilation and then restored to their original size (Fig. 4b).

Localization of hemorrhages

The method of localization of hemorrhages is based upon the combination of mathematical morphological operations and image enhancement techniques. Hemorrhages, which are dark spots in fundus image, are brought to foreground by taking complement of the output (Fig. 5a) of the preprocessing stage defined in section "Preprocessing". The intensity values are adjusted using gamma correction, and this adjustment is with the shape of curve in which mapped values are weighted towards brighter intensities (Fig. 5b). Gamma correction is described by using power-law expression given in Eq. 2.

$$I_{\text{out}} = AI_{\text{in}}^{\gamma} \tag{2}$$

V_{out} is the output image and V_{in} is the input image, where A is constant and γ defines the nature of the gamma curve. In general case, A is 1, which is also true for our proposed method. We did not perform gamma correction on all intensity values present in the image. Intensity values of the input image to be used in gamma correction are clipped between low intensity values IN_{low} and high intensity values IN_{high}. IN_{low} and IN_{high} are selected by saturating the upper 1% and the lower %intensity values present in the input image. The shape of gamma curve was specified using $\gamma = 10$. To remove the blood vessels from the image, the



a Segmented Blood Vessels. **b** Morphological Opening

Fig. 4 Blood vessel segmentation

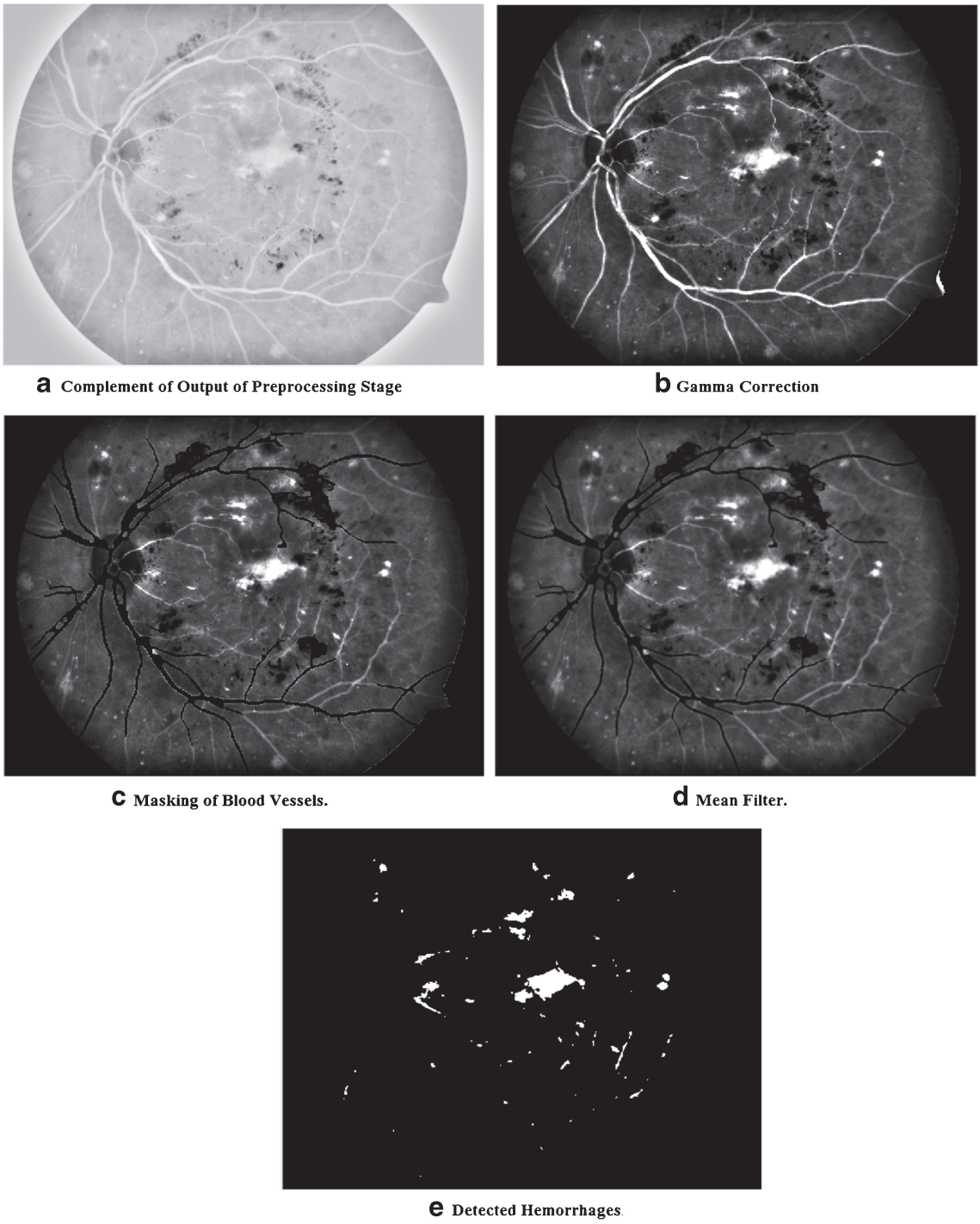


Fig. 5 Localization of Hemorrhages

complement of the detected blood vessels has been taken and is then masked onto the image (Fig. 5c). Mean filter is necessary to smoothen the image after the blood vessels have been segmented out of the image. For calculating the mean filter at the boundaries of the image, intensity values outside the bounds of the image matrix are considered to be equal to the nearest border value (Fig. 5d). The final binary image highlighting the detected hemorrhages is then calculated using global thresholding (Fig. 5e). Thresholding creates binary images from gray-level ones by making all pixels below some threshold to zero and all pixels about that threshold to one. If $f(x, y)$ is the input image,

the image obtained after thresholding $g(x, y)$ is given by Eq. 3 [36].

$$g(x, y) = \begin{cases} 1, \text{ if } f(x, y) \geq t \\ 0, \text{ otherwise} \end{cases} \quad (3)$$

The localization of hemorrhages was performed by using Matlab "im2bw(I, level)" function, where *I* is the input image and *level* is the normalized intensity value in the range [0,1]. This function converts an image into a binary image based on some threshold or level. The output binary image substitutes all pixels in the input image with luminance greater than level with the value 1 (white) and changes all other pixels with the value 0 (black). The proposed model was tested with a number of threshold/level values ranging from 0 to 1 and the value that worked best and yielded the optimal results was found to be 0.47.

Results

The potential of our proposed method has been determined by the performance metrics like sensitivity (SN), specificity (SP), and accuracy as shown in Table 1. For any binary classifier, the output can be termed either as positive or negative. Both outputs again can be either true or false, which gives four different possibilities. If the output of the classifier is positive and the actual value is also positive, it is called as true positive (TP), and if the actual value is negative, this output is termed as false positive (FP). If the output of the classifier is negative and actual value is also negative, it is called as true negative (TN), and if the actual value is positive, this output is termed as false negative (FN).

SN is the ability of an algorithm to detect a pixel as a point of interest. It is the ratio of TP and conditional positive values.

SP is the ability of an algorithm to detect a pixel as a point of the background pixel. It is the ratio of TN and conditional negative values.

The accuracy of the proposed model is estimated by calculating the proportion of true positive and true negative in all evaluated images.

$$\text{Sensitivity(SN)} = \text{TP} / (\text{TP} + \text{FN}) \quad (4)$$

$$\text{Specificity(SP)} = \text{TN} / (\text{TN} + \text{FP}) \quad (5)$$

$$\text{Accuracy} = (\text{TP} + \text{TN}) / (\text{TP} + \text{TN} + \text{FP} + \text{FN}) \quad (6)$$

Our proposed algorithm was tested on DIARETDB1 database and produced SN of 84%, SP of 87%, and an accuracy of 89%. So this demonstrates that our proposed method is capable of producing acceptable and competitive accuracies.

Table 1 Performance comparison of proposed Method with other methods

Method	SN	SP	Accuracy	Approach
Shivaram et al. [26]	89.49%	99.89%	–	Model based
Acharya et al. [31]	82%	86%	–	Model based
Jagatheesh et al. [32]	77.25%	76.40%	85.46%	Model based
Kleawsirikul et al. [33]	80.37%	99.53%	99.12%	Model based
Proposed method	84%	87%	89%	Model based

Conclusions

The computer-aided diagnostic systems based on image processing are becoming wide these days to assist the doctor and also play a potent role in decreasing the diagnostic time. The retinal microcirculation can be viewed non-invasively offering an easily accessible way to study the health and disease of microvasculature in vivo. Improved screening strategies are required for early detection and diagnosing debilitating illnesses such as diabetes. For this purpose, this work proposed an automated method to detect one of the pathological signs of DR. The proposed binary classifier is a model-based approach and does not require any training data sets to classify the input. In this respect, the proposed method is computationally inexpensive and fast. Although supervised methods produce more accurate and quality classification as compared to that of model-based approach; however, they are generally way more computationally expensive. This work can be further extended by fine tuning the results using adaptive thresholding techniques at the final stage and utilizing supervised learning methods.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):88–94
DOI 10.1007/s13410-016-0543-0

ORIGINAL ARTICLE

Comparison of insulin resistance and metabolic syndrome criteria in metabolically obese, normal weight (MONW) individuals in the prediction of cardiovascular disease risk: analysis of the Korean National Health and Nutrition Examination Survey (KNHANES) in 2010–2012

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Received: 14 October 2015 / Accepted: 19 December 2016 / Published online: 27 December 2016

Abstract Body mass index is considered to be insufficient to diagnose obesity in population with metabolic abnormalities. In this study, we aimed to determine the optimal criteria for metabolically obese, normal weight (MONW) with insulin resistance or with metabolic syndrome to provide reliable diagnostic tool for obesity, especially targeting Korean population. This is a cross-sectional study based on the data extracted from the Korean National Health and Nutrition Examination Survey in 2010–2012. A total of 6274 adults with the normal weight were enrolled, and each subject was classified into either MONW with insulin resistance (MONW-IR) or MONW with metabolic syndrome (MONW-Mets) in order to analyze the risk of cardiovascular events. The Framingham risk score (FRS) and atherosclerotic cardiovascular disease risk equation (ASCVD) were used in the process. The odds ratio for the cardiovascular disease risk based on the FRS in the MONW-IR group (1.132; 95% confidence interval (CI), 0.854–1.502) was not significantly elevated whereas the odds ratio for the cardiovascular disease risk using the ASCVD in the MONW-IR group (1.809; 95% CI, 1.410–2.322) was significantly increased. The odds ratio for the cardiovascular disease risk in the MONW-Mets group was both significantly increased (2.93; 95% CI, 2.19–3.91 by FRS, 8.44; 95% CI, 6.19–11.49 by ASCVD) as well. However, the risk of cardiovascular disease was not significantly increased after excluding the subjects with diabetes mellitus that were the majority of MONW-IR group. Metabolic syndrome criteria can be considered more useful tool in diagnosing MONW in the Korean population. However, further prospective studies are needed to confirm our findings.

Keywords Obesity · Metabolic syndrome · Insulin resistance · Cardiovascular risk

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Background

Cardiovascular disease (CVD) is one of the leading causes of death worldwide; thus, it is important to screen and manage the risk factors of CVD [1, 2]. Among the risk factors, obesity diagnosed based on the body mass index (BMI) is considered to be the most essential factor to control in primary prevention [3] despite the fact that BMI is limited in terms of describing exact distribution of body fat [4].

In particular, Asian population possesses many characteristic differences such as subtypes of obesity and the relatively high proportion of body fat compared to the other ethnic groups, even after adjusting variables such as age, sex, and BMI [5, 6]. Moreover, the prevalences of type 2 diabetes and CVD are relatively higher in the Asian than population compared to that in Caucasians [7], hence gave rise to the need for developing a new diagnostic concept. Because, BMI has a limitation as mentioned earlier, Ruderman et al. [8, 9] reported a new concept called "metabolically obese, normal weight (MONW)," which is the subgroup of obesity. The term MONW involves various conditions including insulin resistance, hyperinsulinemia, and dyslipidaemia, and it is thought to be a useful standard for refining obesity in Asian populations [10]. There are several diagnostic criteria of MONW [11–13], and the prevalence of MONW differs according to each criterion. Therefore, it is necessary to adopt the most optimal criterion in order to properly diagnose MONW and to evaluate the risk of cardiovascular events.

Metabolic syndrome is a well-known diagnostic criteria of metabolic abnormalities, that is generally accepted as the diagnostic criteria of MONW. However, it is not suitable for the detection of early forms of MONW. Since the metabolic syndrome is a phenotype of insulin resistance, the ultimate prognosis of patients with insulin resistance would be the development of metabolic syndrome.

In this research, we compared the validity of both insulin resistance and a criterion of metabolic syndrome to predict the future risk of cardiovascular events and to find a proper criteria for MONW in Korean population [14]. We investigated insulin resistance as a diagnostic tool for early forms of MONW and its superiority over metabolic syndrome as an indicator of cardiovascular events. The Framingham risk score (FRS) and the atherosclerotic cardiovascular disease (ASCVD) risk equation described in the new American College of Cardiology/American Heart Association (ACC/AHA) guideline are used for assessing the risk of cardiovascular in the process of verifying validity of each criterion.

Methods

Study subjects

The Korean National Health and Nutrition Examination Surveys (KNHANES) IV and V were two surveys conducted by the Division of Chronic Disease Surveillance, Korean Centers for Disease Control and Prevention, from 2007 to 2010. A stratified, multistage, probability sampling design was applied to make the data nationally representative. KNHANES IV was conducted from July 2007 to December 2009, and individuals older than 1 year from 2300 to 4600 households were included, respectively. KNHANES V was conducted in 2010 with individuals older than 1 year from 3840 households. The data were comprised of the responses from the Health Interview Survey, Health Examination, and Nutrition Survey [15].

The present study included 6274 adults aged 40–79 years who had normal weight. Of these, 159 and 958 subjects had insulin resistance and metabolic syndrome, respectively. This study analyzed data that are available in public, therefore, exempted from institutional review board approval.

Definition of metabolic abnormality

(1) Insulin resistance: homeostasis model assessment of insulin resistance index (HOMA-IR) was used as an indicator of insulin resistance, as follows: [16]

$$\text{HOMA-IR} = (\text{fasting insulin}\,[\mu\text{IU/mL}] \times \text{fasting plasma glucose}\,[\text{mmol/L}])/22.5.$$

Insulin resistance was considered when the HOMA-IR value was in the highest quartile [17].

(2) Metabolic syndrome: The diagnosis of metabolic syndrome was made according to the modified Adult Treatment Panel (ATP) III, proposed by the American Heart Association and National Heart, Lung, and Blood Institute 2005, based on the criteria of the National Cholesterol Education Program-ATP III in 2001 [18]. Abdominal obesity was defined as a waist circumference of ≥90 cm in men and ≥85 cm in women, according to the Korean criteria of abdominal obesity [19]. Metabolic syndrome was diagnosed when at least three of the following criteria were met:

(1) Waist circumference ≥90 cm in men and ≥85 cm in women,
(2) Systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg, or antihypertensive medication use,
(3) Fasting plasma glucose ≥100 mg/dL or antidiabetic medication use,
(4) Triglyceride ≥150 mg/dL or antidyslipidemic medication use, and

(5) High-density lipoprotein (HDL) cholesterol <40 mg/dL in men and <60 mg/dL in women, or antidyslipidemic medication use.

Diagnostic criteria of MONW

(1) MONW on the basis of insulin resistance (MONW-IR) was considered in individuals with normal weight (18.5 kg/m2 ≤ BMI < 23.0 kg/m2) [20] who belonged to the highest quartile of HOMA-IR, excluding those who met the metabolic syndrome criteria described above.
(2) MONW on the basis of metabolic syndrome (MONW-Mets) was considered in individuals with normal weight (18.5 kg/m2 ≤ BMI < 23.0 kg/m2) who met the metabolic syndrome criteria mentioned above, excluding those who belonged to the highest quartile of HOMA-IR.

CVD risk

We used two different cardiovascular risk assessment tools of FRS and ASCD to compare the two criteria of MONW [21, 22].

(1) FRS
In order to estimate the 10-year cardiovascular risk by the FRS, age, smoking status, total and HDL cholesterol level, and systolic blood pressure of the subjects were calculated. The individuals with an FRS of ≥10% were considered to have a high risk of cardiovascular events.
(2) ASCVD risk
The ASCVD risk was obtained using the pooled cohort equations suggested by the 2013 ACC/AHA guideline. Sex, age, ethnicity, total and HDL cholesterol levels, systolic blood pressure, prevalence of hypertension and diabetes, and smoking status of the subjects were used. In this study, individuals with an ASCVD score of ≥7.5% were considered to be at the high risk of having cardiovascular events [22].

Statistical analysis

For weighting all values, complex sample analysis was used according to the statistics from the Korea Centers for Disease Control and Prevention for the KNHANES data. Age, waist circumference, BMI, systolic blood pressure, diastolic blood pressure, total cholesterol level, HDL cholesterol level, triglyceride level, and fasting plasma glucose level for all the participants and for each group were analyzed by using the *t* test. The smoking and physical activity statuses were analyzed using the $\chi 2$ test. Logistic regression analysis was used to determine the association between MONW and cardiovascular risk. For MONW-IR, the lowest quartile of HOMA-IR was used as the reference. All data were analyzed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) while accounting for the complex sampling design.

Results

Baseline characteristic

A total of 159 and 958 subjects were classified into either MONW-IR or MONW-Mets. Age, waist circumference, systolic and diastolic blood pressures, and HDL cholesterol and triglyceride levels of the male subjects were found to be significantly different between MONW-IR and MONW-Mets groups, while the age, hypertension, waist circumference, and systolic and diastolic blood pressures of the female subjects were significantly different between the two groups.

In the intragroup comparison according to sex, the prevalence of diabetes mellitus (DM), waist circumference, and smoking status were found to be significantly different in the MONW-IR group; however, the prevalence of hypertension and DM, waist circumference, diastolic blood pressure, triglyceride, total cholesterol, aspartate amino-transferase, alanine transaminase, and smoking status were significantly different between men and women in the MONW-Mets group (Table 1).

Odds ratios for CVD risk according to each MONW criterion

We estimated the risk of cardiovascular events according to the different MONW criteria using the FRS and ASCVD risk equation. For subjects classified as MONW-IR, the risk of cardiovascular events using the FRS did not significantly differ (odds ratio [OR], 1.132; 95% confidence interval [CI], 0.854–1.502) compared to the subjects without insulin resistance; however, the risk of cardiovascular events using the ASCVD equation was significantly increased (OR, 1.809; 95% CI, 1.410–2.322) in patients with insulin resistance compared to subjects without insulin resistance. For MONW-Mets, the odds ratio estimated by using both FRS (OR, 2.93, 95% CI, 2.19–3.91) and ASCVD (OR, 8.44; 95% CI, 6.19–11.49) for the risk of cardiovascular events was significantly increased, compared to that of patients without metabolic syndrome, and this was more prominent in women (Table 2).

Odds ratio for CVD risk in MONW-IR, excluding those with DM

The prevalence of DM in the MONW-IR group was 84.8% in men and 54.4% in women (Table 1). After excluding individuals with DM from the MONW-IR group, the risk of CVD

Table 1 Baseline characteristic according two diagnostic criteria and sex

Characteristics	MONW-IR (159)			MONW-Mets (958)			*P* value†	
	Male (69)	Female (90)	*P* value*	Male (487)	Female (576)	*P* value*	Male	Female
Age	53.27 ± 1.39	53.00 ± 1.39	0.892	58.38 ± 1.03	62.62 ± 0.81	0.001	0.001	<0.001
HTN (%)	16.3	11.6	0.444	27.0	38.4	0.039	0.058	<0.001
DM (%)	84.8	54.4	<0.001	77.3	64.0	0.019	0.212	0.132
WC	78.50 ± 0.79	75.00 ± 0.67	0.001	82.21 ± 0.52	77.81 ± 0.51	<0.001	<0.001	0.006
BMI	21.36 ± 0.18	21.41 ± 0.15	0.819	21.67 ± 0.09	21.59 ± 0.08	0.517	0.297	0.278
SBP	121.55 ± 2.02	116.67 ± 2.00	0.081	129.13 ± 1.71	127.70 ± 1.55	0.532	<0.001	<0.001
DBP	76.99 ± 1.24	73.25 ± 1.19	0.044	81.38 ± 0.97	77.64 ± 0.92	0.007	<0.001	<0.001
HDL-C	54.26 ± 2.01	52.37 ± 1.13	0.399	41.25 ± 0.86	42.34 ± 0.71	0.332	<0.001	<0.001
Triglyceride	101.61 ± 6.44	99.88 ± 4.80	0.826	234.38 ± 15.43	197.35 ± 9.87	0.040	<0.001	<0.001
Total-C	183.90 ± 5.62	194.37 ± 4.61	0.151	195.11 ± 3.14	205.66 ± 3.10	0.013	0.354	0.089
Fasting glucose	125.66 ± 5.76	119.50 ± 5.62	0.435	113.78 ± 2.83	109.40 ± 2.15	0.217	0.097	0.188
AST	36.17 ± 7.56	21.33 ± 1.36	0.053	30.91 ± 3.32	23.45 ± 1.11	0.034	0.532	0.208
ALT	42.00 ± 15.05	23.53 ± 5.15	0.248	28.28 ± 2.15	22.25 ± 1.57	0.025	0.374	0.614
Smoking, %	80.2	14.6	<0.001	87.9	10.0	<0.001	0.339	0.322
Physical activity‡(%)	15.6	16.6	0.883	22.4	17.0	0.255	0.304	0.869

Values are expressed as mean ± SD or frequency (%)

Abbreviation: *MONW-IR* metabollicaly obese normal weight diagnosed by insulin resistance, *MONW-Mets* metabolically obese normal weight diagnosed by metabolic syndrome, *HTN* hypertension, *DM* diabetes mellitus, *WC* waist circumference, *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *HDL-C* high density lipoprotein cholesterol, *Total-C* total cholesterol, *AST* aspartate aminotransferase, *ALT* alanine animotrasferase

**P* value: intragroup comparison according to sex, †*p* value: intergroup comparison according to sex, ‡physical activity: moderate exercise more than three times a week

using the ASCVD risk equation was not significantly increased (Table 3).

Discussion

Managing obesity is one of the main goals of public health interventions in many countries, including Korea [23, 24]. In this study, we compared two different criteria of MONW. In order to determine the validity of such comparison, we classified enrolled subjects into two groups which were not overlapped and adopted FRS and ASCVD for the calculations.

According to our study, the cardiovascular risk of MONW-IR calculated by the ASCVD equation significantly increased, but the result has been different when FRS was applied. Also, the risks based on ASCVD and FRS were not increased after the subjects with diabetes mellitus in MONW-IR were excluded. On the other hand, the cardiovascular risk was

Table 2 Odds ratio of high cardiovascular risk according to two diagnostic criteria of MONW

High cardiovascular risk		MONW-IR Odds ratio (95% CI)[a]				MONW-Mets Odds ratio (95% CI)[a]
		Q1	Q2	Q3	Q4	
FRS	All	1.00 (reference)	0.907 (0.725–1.135)	0.734 (0.558–0.965)	1.132 (0.854–1.502)	2.93 (2.19–3.91)
	Male	1.00 (reference)	1.038 (0.731–1.474)	1.185 (0.737–1.903)	1.164 (0.744–1.819)	3.538 (2.212–5.817)
	Female	1.00 (reference)	1.460 (0.748–2.850)	0.604 (0.223–1.638)	1.906 (0.906–4.010)	13.866 (9.195–20.911)
ASCVD risk	All	1.00 (reference)	1.108 (0.902–1.360)	0.956 (0.731–1.249)	1.809 (1.410–2.322)	8.44 (6.19–11.49)
	Male	1.00 (reference)	1.212 (0.890–1.650)	1.454 (0.919–2.300)	2.611 (1.694–4.024)	6.746 (4.320–10.533)
	Female	1.00 (reference)	1.323 (0.979–1.788)	0.965 (0.650–1.432)	1.689 (1.095–2.604)	11.876 (9.036–15.609)

Abbreviation: *MONW-IR* metabollicaly obese normal weight diagnosed by insulin resistance, *MONW-Mets* metabolically obese normal weight diagnosed by metabolic syndrome, *CI* confidence interval, *Q1* lowest quartile of HOMA-IR, *Q2* second quartile of HOMA-IR, *Q3* third quartile of HOMA-IR, *Q4* highest quartile of HOMA-IR, *FRS* Framingham risk score ≥10%, *ASCVD risk* Atherosclerotic cardiovascular risk ≥7.5%

[a] *p* value by logistic regression using complex sample

Table 3 Odds ratio of high ASCVD risk according to MONW-IR excluding DM

High cardiovascular risk	MONW-IR excluding DM (95% CI)[a]				
		Q1	Q2	Q3	Q4
ASCVD risk	all	1.00 (reference)	1.020 (0.815–1.277)	0.821 (0.601–1.121)	1.034 (0.701–1.524)
	Male	1.00 (reference)	1.078 (0.772–1.507)	1.188 (0.706–2.001)	1.631 (0.879–3.027)
	Female	1.00 (reference)	1.281 (0.930–1.764)	0.823 (0.530–1.277)	1.085 (0.542–2.173)

Abbreviation: *MONW-IR* metabollicaly obese normal weight diagnosed by insulin resistance, *DM* diabetes mellitus, *CI* confidence interval, *Q1* lowest quartile of HOMA-IR, *Q2* second quartile of HOMA-IR, *Q3* third quartile of HOMA-IR, *Q4* highest quartile of HOMA-IR, *ASCVD risk* Atherosclerotic cardiovascular risk ≥7.5%

[a] Analyzed by logistic regression using complex sample

significantly increased when estimated by both the FRS and ASCVD risk equation in MONW-Mets.

In the current study, the prevalences of diabetes mellitus and abdominal obesity were both significantly higher in men than in women for both groups, corresponding directly to the result established in the previous researches in which the risk of type 2 diabetes mellitus is significantly higher in men than in women in middle age [25, 26]. It was also reported that prevalence of abdominal obesity in Korean population was 25.0% in men and 21.1% in women, respectively, [27] which reflects the different amounts of insulin resistance between genders. Men are less insulin sensitive than women, indicating the distribution of fat occurs very differently in each sex. In men, the ability to increase subcutaneous fat storage is less effective than that of women; hence, as weight increases, excess fat is more likely to be stored in locations other than subcutaneous layer. This so-called ectopic fat appears to be an accelerator for increasing the waist circumference [28].

In this study, the risk of cardiovascular events calculated by ASCVD risk equation was both significantly high in MONW-IR and MONW-Mets. According to previous studies which compared a group of normal weight with insulin resistance (IR-NW) with a group of obesity without insulin resistance (insulin sensitivity, IS-obese) [14], the risk for cardiovascular events was found to be significantly higher in the IR-NW group compared to IS-obese group (hazard ratio, 1.98; 95% CI, 0.86–4.54). However, the fact that subjects with diabetes were not excluded in the study, even though the number was relatively small, differentiates our study from the previous researches. Another former study, designed to assess the degree of arterial stiffness and carotid atherosclerosis in metabolically abnormal but normal weight (MANW) group and metabolically healthy but obese (MHO) individuals, supports the results of our research by reporting that metabolic syndrome is likely to increase the risk of developing atherosclerosis in the normal weight group [29].

In this study, after excluding patients with diabetes mellitus from the MONW-IR group, the risk of cardiovascular events was not significantly increased (odds ratio 1.034, 95% CI, 0.701–1.524). Metabolic syndrome is the well-known phenotype of insulin resistance [30], which means that insulin resistance without metabolic syndrome is considered the state of subclinical metabolic abnormality. Hence, it is crucial to assess the validity of insulin resistance as a single reliable indicator which leads to diagnosis of the MONW. Insulin resistance is regarded as a significant predictive factor used to assess the risk of cardiovascular event as calculated earlier in the paper based on both the FRS and ASCVD risk equation [23, 31] and is the main pathogenesis of diabetes mellitus [32]. However, discussing all the possibilities, insulin resistance is not the only indicative factor which augments the risk of cardiovascular event. For instance, insulin resistance observed without diabetes mellitus or metabolic syndrome tends not to increase the risk over all. Therefore, it is possible to conclude that the validity of insulin resistance alone as a diagnostic tool of MONW is not as effective as metabolic syndrome criteria, especially in patients with subclinical state.

In this study, the cardiovascular risk determined using the ASCVD risk equation was significantly increased in both MONW-IR and MONW-Mets, while the cardiovascular risk based on the FRS was significantly increased only in MONW-Mets. Besides, the ASCVD risk equation was found to indicate a higher cardiovascular event in elders (60–75 years) compared to that of younger (40–59 years) subjects, and the same exact patterns were observed in men versus women and individuals with higher blood pressure versus those with normal blood pressure [33]. Furthermore, in this study, the proportions of elderly participants aged over 60 years old were 40.7 and 53.8%, respectively, in the MONW-IR and MONW-Mets groups. The proportion of elderly subjects was considerably high, and this can be an explanation for the high risks of cardiovascular event calculated in the process. Higher odds ratio in MONW-IR compared to subjects without insulin resistance by ASCVD equation may be able to create selection bias. Although it is known that insulin resistance increases the risk of cardiovascular event, insulin resistance alone as was less effective tool for diagnosing MONW in Korea.

This study has several strengths. First, we analyzed data representing general Korean population; 159 of MONW-IR and 958 of MONW-Mets were weighted to be equivalent to

1.09 million and 5.08 million, respectively. Furthermore, to our knowledge, it is the first study designed to compare the validity of each criterion of MONW by assessing cardiovascular event risk. Second, we compared metabolic syndrome and insulin resistance independently by excluding cases that fall into both categories at the same time; therefore, we could observe the correlation between each criterion and cardiovascular risk more effectively. Lastly, we used both models simultaneously to overcome the limitations reside in each individual equation model used for predicting CVD risk.

There are some limitations as well. First, we used the FRS and ASCVD risk equation instead of direct cardiovascular exams to assess the cardiovascular risk. It has been suggested that these two models tend to overestimate the risk of CVD [23], and calculations based on FRS yield results that have been reported to be especially high in Asians [34]. Second, this study was designed to be a cross-sectional study; thus, we could only determine the correlation among factors, but not among the causalities. Finally, we used HOMA-IR for estimating insulin resistance. Although HOMA-IR can precisely detect insulin resistance in large populations [16, 35], it may be inaccurate in estimating insulin resistance in individuals [36]. Despite of those limitations, we believe that the results of this study will be useful in various clinical settings for evaluating MONW.

Conclusions

In conclusion, our findings indicate insulin resistance alone is insufficient for diagnosing MONW. Since this study was based on cross-sectional manner, further prospective studies are required to fully establish diagnostic criteria of MONW.

Authors' contributions HR H and DW J collected data and drafted the manuscript. YJ K, SY L, JG L, YH K, YH Y, YH C, YJ T, and AZ performed the statistical analysis and drafted the manuscript. All authors read and approved the final manuscript

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):95–99
DOI 10.1007/s13410-017-0551-8

ORIGINAL ARTICLE

Association of risk factors with a major re-amputation in Malaysian diabetic patients: a retrospective cohort analysis of patient registry

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Received: 1 June 2016 / Accepted: 21 February 2017 / Published online: 1 March 2017

Abstract This study aimed to determine the association of risk factors with a major re-amputation in Malaysian diabetes patients. This is a retrospective review and analysis of a patient cohort obtained from National Orthopedic Registry Malaysia (NORM) between June 2008 and December 2009. Re-amputation is defined as the removal of bones to advance to a higher level of amputation. An amputation can be regarded as a re-amputation if it is performed on the same limb within the same admission period and is also considered to be the final level of amputation. The plausible risk factors that are reported as importantly associated with the risk of re-amputation include patients' baseline socio-demographic factors, clinical characteristics, and treatment outcomes, and these are analyzed both separately and together. A total of 137 patients were eligible for inclusion into the study. Only 23.4% of the total 137 patients were actually re-amputated. Baseline clinical characteristics such as the presence of neuropathy (p = 0.004), nephropathy (p = 0.005), and the topical application of emollient (p = 0.003) are found to be associated with requirement for a major re-amputation. Almost a quarter of patients who had an initial amputation will require a re-amputation. The patients who were at a particularly high risk of the need for re-amputation were those experiencing complications of diabetes such as nephropathy. Topical application of emollient is necessary to delay the need for re-amputation and if possible, avoid it altogether.

Keywords Diabetes · Re-amputation · Risk factors

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Introduction

Foot lesions with ulcer formation leading to limb amputation are a major problem in a diabetic patient. People with diabetes have at least 15% higher risk of having to undergo a lower-extremity amputation (LEA) than those without diabetes [1]. Amputation is often a surgical procedure to be used as the last resort, and it will only be performed to improve the quality of life of patients who still have a salvageable limb. In a diabetic patient, a major limb amputation can be associated with a high risk of contralateral re-amputation [2], together with its associated morbidity and mortality [2–4].

The number of people suffering from diabetes is expected to continue to increase from 366 million in year 2011 to 552 million by year 2030. It is likely that there are as many as 183 million people who are totally unaware that they have diabetes [5]. Diabetes is a debilitating disease and the associated complications can lead to significant increases in both mortality and morbidity. Once diagnosed, it is important that diabetic patients are promptly given adequate treatment for preventing or delaying the disabling complications of diabetes. The feet of diabetic patients are at a particularly high risk, mainly because the way which the disease process of diabetes can affect bodily sensation, its blood flow and its ability to fight infections. Neuropathy in patients with diabetes was also a contributing risk factor for the development of foot ulcer. Peripheral vascular disease in a diabetic

patient was associated with a threefold increased risk of the need for lower limb amputation [6].

A review of the current literature has found the reported rates of re-amputation in diabetic patients whose data come from the patient populations in the developed nations. There is a paucity of current literature on the reported rates of re-amputation in diabetic patients within the Asia countries. Currently, there are no available data from Malaysia and the other ASEAN countries. Therefore, from the data gathered in this research paper, we hope that the clinicians and primary service providers will now be able to modify the risk factors of these diabetic patients by providing effective treatment continuously which shall lead to better functional outcomes for those who had a prior history of an initial limb amputation.

Methodology

This was a retrospective cohort review and analysis of patient data obtained from the National Orthopaedic Registry of Malaysia (NORM). There are a total of 18 Ministry of Health (MOH) Malaysia hospitals providing orthopedic specialist services which are currently contributing data to this disease registry. Each of these hospitals has a team of dedicated personnel (i.e., research assistants, nurses, or medical officers) to fill in an online electronic case report form (eCRF) which shall be used as an assessment tool.

Data obtained from all diabetic patients with complications of their feet or hands who were admitted into one of the 18 hospitals for treatment between June 2008 and December 2009 were included in this patient registry. Since this study aims to identify risk factors which are associated with a major re-amputation in Malaysia, therefore, all diabetic patients who had a history of an initial limb amputation in the previous 1 year were included. A minor amputation will usually involve the toes and feet only, while a major amputation will usually involve the part of the leg above the ankle. Re-amputation is defined as the removal of bones to advance to a higher level of amputation. An amputation can be regarded as a re-amputation if it is performed on the same limb within the same admission period and is also considered to be the final level of amputation.

The criteria for neuropathy were evaluated by using the Semmes-Weinstein monofilament test for both feet at time of admission. Using the 10 g/5.07 monofilament at ten different sites of the feet performed this test. The site which was not felt by the monofilament was put "negative" and "positive" for the site which still has sensation. For diabetic nephropathy and retinopathy, the data were mainly gathered from their medical history.

All human studies have been reviewed by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki. There is no individual consent was taken since this study used a patient registry database. However, an approval was obtained from Medical Research and Ethics Committee with registration number NMRR-08-1349-2597.

Statistical analysis

The summary of descriptive statistics for age was presented as means and standard deviations (SD). All the categorical variables were presented as frequencies and percentages. Cross tabulation with Pearson's chi-square test was performed to test whether (or not) there is an association between patients' baseline socio-demographic factors, their diabetic treatment regimens, presence of precipitating factors for the need for re-amputation, presence of complications of diabetes, and other co-morbidities between the two groups of patients (i.e., one group of patients undergoing re-amputation and the other group of patients not undergoing re-amputation). Logistic regression was then applied for multivariate analysis. The adjusted odds ratio with their respective 95% confidence intervals were calculated after controlling for possible confounders such as age, gender, and race in the analysis. All statistical analyses were carried out using IBM SPSS version 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.)

Results

A total number of 1886 patients were included in this patient registry database of patients with diabetic foot and hand diseases for the period of time between June 2008 and December 2009. From this group of 1886 patients, a total of 137 patients with a history of previous amputation were included in this study. The status of re-amputation was classified as either "re-amputation group" or "non re-amputation group." Thirty-two (i.e., 23.4%) of them had undergone a re-amputation, which forms the "re-amputation group." The remaining 105 patients who did not require any further amputation will form the "non re-amputation group." The patients in the "re-amputation group" were slightly older with a mean (SD) age of 60.2 (8.0) years. There was no significant association between patients' baseline socio-demographic factors and status of re-amputation (as shown by Table 1).

Co-morbidity and diabetes complications

There was a statistically significant positive association between nephropathy and re-amputation (i.e., $p = 0.005$ for univariate analysis and $p = 0.025$ for multivariate analysis), which means that it is likely that a higher degree of nephropathy will lead to a greater need for re-amputation. However, there was a statistically significant negative association

Table 1 Association between demographic profiles of diabetic foot patients toward status of re-amputation

	No re-amputation *n* (%)	Re-amputation *n* (%)	*P* value
Age group[a]	56.9 (8.7)	60.2 (8.0)	0.061
Gender			
Male	51 (48.6)	18 (56.3)	0.447
Female	54 (51.4)	14 (43.8)	
Race			
Malay	80 (76.2)	22 (68.8)	0.105
Chinese	8 (7.6)	7 (21.9)	
Indian	13 (12.4)	3 (9.4)	
Other	4 (3.8)	0 (0)	
Education level			
Nil	7 (6.8)	2 (6.3)	0.526
Primary	31 (29.5)	9 (28.1)	
Secondary	37 (37.8)	7 (21.9)	
Tertiary	5 (4.8)	2 (6.3)	
Not known	25 (23.7)	12 (37.5)	
Duration of diabetes			
≤1 year	3 (3.3)	0 (0.0)	0.757
2–5 years	16 (17.8)	5 (17.9)	
6–10 years	27 (30.0)	10 (35.7)	
>10 years	44 (48.9)	13 (46.4)	
Type of diabetes mellitus			
T1DM	17 (16.2)	2 (6.3)	0.154
T2DM	88 (83.8)	30 (93.8)	

[a] Reported in mean and standard deviation (SD)

Table 2 Association between patients' co-morbidity and diabetes complications toward status of major re-amputation

	No re-amputation *n* (%)	Re-amputation *n* (%)	*P* value
Co-morbidity and complications			
Hypertension			
No	41 (39.0)	11 (34.4)	0.633
Yes	64 (61.0)	21 (65.6)	
Hyper cholesterol			
No	88 (83.8)	30 (93.8)	0.154
Yes	17 (16.2)	2 (6.2)	
Ishaemic heart disease			
No	87 (82.9)	26 (81.2)	0.834
Yes	18 (17.1)	6 (18.8)	
Cerebrovascular			
No	99 (94.3)	28 (87.5)	0.196
Yes	6 (5.7)	4 (12.5)	
Retinopathy			
No	72 (68.6)	27 (84.4)	0.080
Yes	33 (31.4)	5 (15.6)	
Vascular inefficiency			
No	90 (85.7)	29 (90.6)	0.472
Yes	15 (14.3)	3 (9.4)	
Neuropathy			
No	59 (56.2)	27 (84.4)	0.004
Yes	46 (43.8)	5 (15.6)	
Nephropathy			
No	91 (86.7)	23 (71.9)	0.005
Yes	14 (13.3)	9 (28.1)	

between neuropathy and re-amputation (i.e., $p = 0.004$ for univariate analysis and $p = 0.040$ for multivariate analysis), which means that it is likely that a higher degree of neuropathy will lead to a lesser need for re-amputation. It was also found that there was no statistical significance for the association between the other co-morbidities and complications of diabetes, and re-amputation. Results obtained from multivariate analysis had shown that patients with nephropathy are at least four times more likely to be at risk for re-amputation (Tables 2 and 4).

Treatment and awareness

It was found that there is a negative association between the practice of using topical emollient in the feet and re-amputation (i.e., $p = 0.003$ for univariate analysis and $p = 0.018$ for multivariate analysis). We currently still do not have sufficient evidence to identify an association between treatment of diabetes and re-amputation (Table 3). Results obtained from multivariate analysis had shown that patients who did not apply topical emollient in the feet are at least four times more likely to be at risk for re-amputation (Tables 3 and 4).

Types of re-amputations

The most common types of re-amputation were ray (16/35 = 45.7%) and below knee (14/35 = 40.0%), which were then followed by disarticulation ($n = 2$), through knee ($n = 2$), and syme ($n = 1$).

Discussion

Rate of re-amputation in our Malaysian patient population was 23.4%. This rate was almost the same as that in Taiwan (i.e., 23.3%) [7]. In the UK, the rate of re-amputation had ranged from 4 to 30% [8]. Another study reported the rate of re-amputation over time, such as 10.4% after 1 month, 16.5% after 3 months, 18.8% after 6 months, and 23.1% after 4 years [9]. In general, the rate of re-amputation had reached almost as high as one quarter among the amputees and would still be increasing in tandem with the duration of having diabetic condition.

It is already known that complications of diabetes such as peripheral vascular disease, neuropathy, and infection are the main contributing factors of amputation [10].

Table 3 Association between treatment and awareness of diabetes foot toward status of re-amputation

	No re-amputation *n* (%)	Re-amputation *n* (%)	*P* value
Treatment			
Diet			
Yes	22 (21.0)	8 (25.0)	0.628
No	83 (79.1)	24 (75.0)	
Insulin only			
Yes	26 (24.8)	7 (21.9)	0.738
No	79 (75.2)	25 (78.1)	
OHA only			
Yes	54 (51.4)	13 (40.6)	0.284
No	51 (48.6)	19 (59.4)	
OHA and insulin			
Yes	20 (19.1)	6 (18.8)	0.970
No	85 (81.0)	26 (81.3)	
Practices (daily/weekly/occasionally)			
Practice inspect			
Feet/hand			
Yes	62 (69.7)	16 (57.1)	0.220
No	27 (30.3)	12 (42.9)	
Practice wash feet			
Yes	70 (76.1)	18 (62.1)	0.139
No	22 (23.9)	11 (37.9)	
Practice apply emollient			
Yes	42 (46.2)	4 (14.8)	0.003
No	49 (53.8)	23 (85.2)	
Attend foot clinic			
Yes	35 (40.7)	8 (28.6)	0.250
No	51 (59.3)	20 (71.4)	

Table 4 Associated factors toward re-amputation: a multivariate analysis

Factors	Adjusted OR	95%CI	*P* value
Neuropathy			
Yes	0.259	0.071, 0.942	0.040
No	Reference group		
Nephropathy			
Yes	4.276	1.200, 15.235	0.025
No	Reference group		
Apply emollient (daily/weekly/occasionally)			
Yes	Reference group		
No	4.326	1.283, 14.586	0.018

Result was derived after controlled for gender, age, and race in the analysis

However, there are very few studies which examine the roles of risk factors of the need for re-amputation. A study found that both the pre-existing gangrene upon admission and a history of insulin-dependent diabetes were significant risk factors of the need for re-amputation [11]. Another study found that both an increasing age and the level of amputation [12] were positively associated with re-amputation. Generally, the patient will be at a greater risk of the need for re-amputation if the diabetic condition is more severe.

This study found that nephropathy is a major contributing factor of the need for re-amputation. Nephropathy is one of the major complications of diabetes which usually occur in older patients having a longer duration of the disease [13]. Since nephropathy is a major contributing factor of the need for re-amputation, hence, it is important to prevent this complication of diabetes. Therefore, it is recommended to always maintain a close monitoring of blood glucose, lipid profile, and blood pressure in the older diabetic patients to prevent kidney damage resulting from diabetes [14].

On the other hand, it was found that there was a statistically significant negative association between neuropathy and re-amputation, which means that it is likely that a higher degree of neuropathy will lead to a lesser need for re-amputation. This is in contrast to those previous findings which found that neuropathy is one of the main contributing factors of the need for amputation [15–17]. However, this study had shown that neuropathy is no longer a major contributing factor of the need for re-amputation among the amputees. In fact, it was found that there was a negative association between neuropathy and re-amputation, which means that it is likely that a higher degree of neuropathy will lead to a lesser need for re-amputation. It is difficult to explain why this occurs, especially when there are only very few studies reporting on the risk of the need for re-amputation. However, one possible reason which we can postulate now is that among those patients with neuropathy, amputation has already been done at a higher level so that it shall be less likely to require re-amputation at a lower level. Unfortunately, we do not have data to support this statement and so we shall suggest future research studies to look into this.

This study had shown that it is likely that the practice of applying topical emollient to prevent dry skin and subsequent skin infection shall reduce the risk of the need for re-amputation. Severe skin dryness if not been treated properly will lead to the development of diabetic ulcers [18]. Since skin infection is a major contributing factor of the need for amputation and also re-amputation, hence, the topical application of emollient is necessary to prevent dry skin and subsequent skin infection. From our data, it was found that patients who did not use topical emollient was four times more likely to be at risk of the need for re-amputation. Thus, it is recommended for all diabetic patients as a self-care measure.

This study has few limitations. This study was not able to provide baseline characteristics with regard to laboratory findings to understand the status on patients' glycemic status and kidney function because these variables are not been included or been maintained in this registry. Future studies may consider to test the association of these factors toward re-amputation.

The conclusion of this research paper shall sum up by stating that severe complications of diabetes such as nephropathy can be positively associated with the risk of need for re-amputation. Thus, this group of diabetic patients having severe complications of diabetes such as nephropathy should be closely monitored since the they are mostly elderly and are also at a greater risk of the need for re-amputation. Topical application of emollient on the skin surfaces is highly recommended to prevent skin infection and subsequently the need for a re-amputation.

Acknowledgements We would like to extend the appreciation to Director General of Ministry of Health for his support to publish the work. We thank also to the National Orthopedic Registry Malaysia for providing the data and also to Mr. John Hon Yoon Khee for his effort in proofreading this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study involved human participants and performed in accordance with the ethical standards of Medical Research and Ethics Committee with registration number NMRR-08-1349-2597 and in accordance with the 1964 Declaration of Helsinki.

Informed consent Informed consent was not obtained from individual participants because this study used a patient registry database.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):100–107
DOI 10.1007/s13410-017-0555-4

ORIGINAL ARTICLE

Emergence of multi drug resistance strains causing diabetic foot infection in Salem, Tamil Nadu, India

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Received: 23 October 2016 / Accepted: 21 March 2017 / Published online: 20 April 2017

Abstract This study was to assess the emergence of *mecA* and $bla_{CTX\text{-}M}$ in multidrug resistant MRSA/MRSE and ESBL strains in diabetic foot infections. A total of 160 pus samples were collected from the diabetic foot care hospitals, Salem, Tamil Nadu, India. The samples were processed for microbiological investigations and further evaluating the resistant strains. The bacterial strain *Staphylococcus aureus* was the predominant organisms isolated from the sample. Cefoxitin, Oxacillin and Vancomycin were found to be the most effective antimicrobial agents for therapy of gram positive organisms while Meropenem, Piperacillin, Cefoperazone/ Sulbactam, Piperacillin/ Tazobactam and Amikacin were found to be the most effective antimicrobial agents for the gram negative organisms. In this study, four isolates of Methicillin resistant *Staphylococcus aureus* (MRSA) strains, one isolate of Methicillin resistant *Staphylococcus epidermidis* (MRSE) and five isolates of *Pseudomonas aeruginosa* were found to be extended spectrum of β - lactamase (ESBL) producers by phenotypic method. These strains subjected to genotypic confirmation confirmed the presence of resistant genes namely $bla_{CTX\text{-}M}$ in ESBL and SCC *mecA* in *Staphylococcus* spp. The 16S rRNA partial gene sequencing and phylogenetic analysis of the multi drug resistant organisms *Pseudomonas aeruginosa* (APS01) and *Staphylococcus epidermidis* (APS02) was confirmed the resistant pattern in these strains. The resistant organism was eradicated only by the effective management of this infection; knowledge on the causative agent causing cruel diabetic foot infections will be very useful in selecting the appropriate therapy.

Keywords Diabetic foot · *mecA* · $bla_{CTX\text{-}M}$ · Phylogenetic tree

Introduction

Diabetic foot infections are one of the leading causes of morbidity, in the developing countries like India. The foot infection initially begins as a superficial ulcer and proceeds further with the delay in treatment and is extended to the other subcutaneous tissues and finally into deeper structures [1]. This type of clinical pathology is the characteristic of diabetic foot infection. Despite the magnitude of this problem, the research insights are first and foremost focused on the role of potential aetiological agents and factors associated with these infections [2]. Most of these diabetic foot infections are polymicrobial. In recent years, the presence of multidrug resistant organism has further complicated the treatment regimes as well as increased the hospital stay and the cost engaged in the treatment of these patients. In gram positive organisms, the presence of Methicillin resistant *Staphylococcus aureus* (MRSA) and Methicillin resistant *Staphylococcus epidermidis* (MRSE) as one of the most common organisms found in these diabetic foot infections [3]. In gram negative pathogens, beta lactamases remain the most important contributing factor to beta lactam resistance and their increasing prevalence in foot infections. This resistance is mainly attributed to presence of $bla_{CTX\text{-}M}$ gene in its genome. There are signs of the presence of $bla_{CTX\text{-}M}$ genes in the genome of European patients in 1980 [4] but now it has been increasingly reported. In India, there is paucity of data on the frequency of this multidrug resistant organism and the outcome of infections among

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diabetic foot patients [5]. The report from Chandigarh, India has reported 68.5% of their diabetic foot infected isolates were ESBL producers by phenotypic methods [6]. The first report on the molecular detection of ESBL strains from a foot infection was from Aligarh, India [7]. Although there are no studies with regard to the molecular aspects of detection of resistant genes from ESBL and MRSA isolates with regard to this from the southern most part. Hence, this aspect has been dealt with in this study by the emergence of *mecA* and $bla_{CTX\text{-}M}$ in multidrug resistant MRSA/MRSE and ESBL strains in diabetic foot infections.

Materials and methods

General

A total of 160 patients with diabetic foot infections presenting at the outpatient department of the diabetic foot care hospitals, Salem, Tamil Nadu, India were enrolled in this study. The institutional ethical clearance was obtained for enrolment of human subjects for this study (IEC/PU/HR/2012/003). The study participants were given the informed consent form prior to sampling.

Microbiological investigations

After the clinical assessment, the microbiological investigations were carried out from the pus material collected from deeper portions of the ulcer by making a rotatory movement with the swab. Two swabs were used for collecting the samples. The collected samples were promptly transported to the laboratory in a sterile swab container under strict aseptic conditions. The samples were processed by inoculation on to culture media like Brain Heart Infusion Agar (BHIA), Nutrient Agar (NA) and incubated at 37°C for 24 h. The bacterial isolates grown on the media were confirmed with the help of Bergey's Manual of Determinative Bacteriology [8].

In vitro susceptibility pattern

The antimicrobial susceptibility testing of the bacterial isolates was performed as described in CLSI guidelines [9]. The antibacterial disks included Erythromycin (15 μg), Chloramphenicol (30 μg), Clindamycin (10 μg), Vancomycin (30 μg), Tetracycline (30 μg) and Ciprofloxacin (5 μg) for Gram positive organisms. Aztreonam (30 μg), Amoxyclav (30 μg), Cefpodoxime (10 μg), Cefepime (30 μg), Cefoperazone (75 μg), Cefoperazone/ sulbactam (75/10 μg), Cefixime (5 μg), Piperacillin (100 μg), Ceftriaxone (30 μg), Amikacin (30 μg), Rifampicin (5 μg), Meropenem (10 μg), Cefoxitin (30 μg), Ticarcillin/Clavulanic acid (75/10 μg) and Piperacillin/Tazobactam (100/10 μg) for the Gram negative organisms. The strains of *Staphylococcus aureus* MTCC 96 and *Pseudomonas aeruginosa* MTCC 424 (obtained from Microbial Type Culture Collection, Chandigarh, India) were used as control strains.

ESBL confirmatory test

The phenotypic confirmation of the extended spectrum of beta lactamases (ESBL) producing strains was done by the combined disc method as per the CLSI guidelines. Disc of Ceftazidime (30 μg) and Ceftazidime/ Clavulanic acid (30/10 μg) were placed on Muller Hinton agar (MHA) on which 0.5 McFarland of the test isolates was swabbed. The organisms were considered as ESBL producer if there was ≥5 mm increase in zone diameter of Ceftazidime / Clavulanic disc and that of Ceftazidime disc.

Table 1 Antibiotic susceptibility pattern of gram positive organisms from cases of diabetic foot infections

S.No	Name of the Organism	Total No of isolates	Antibiotics (No. of isolates and % of Sensitive)								
			E	AK	C	CD	T	CIP	VA	OX	CX
1	*Staphylococcus aureus*	62	38(61)	54(87)	30(48)	54(87)	54(87)	55(89)	61(98)	58(94)	58(94)
2	*Staphylococcus saprophyticus*	21	14(67)	20(95)	20(95)	20(95)	19(90)	20(95)	21(100)	21(100)	21(100)
3	*Bacillus subtilis*	11	2(18)	11(100)	2(18)	11(100)	11(100)	11(100)	11(100)	11(100)	11(100)
4	*Staphylococcus epidermidis*	8	7(88)	7(88)	7(88)	7(88)	7(88)	7(88)	-	7(88)	7(88)
5	*Micrococcus luteus*	6	2(33)	6(100)	2(33)	6(100)	2(33)	6(100)	6(100)	6(100)	6(100)
6	*Streptococcus pneumoniae*	2	1(50)	2(100)	2(100)	2(100)	1(50)	2(100)	2(100)	2(100)	2(100)
7	*Listeria monocytogens*	1	-	1(100)	-	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)

*E - Erythromycin; AK - Amikacin; C - Chloramphenicol; CD - Clindamycin; T - Tetracycline; CIP - Ciprofloxacin; VA - Vancomycin; OX - Oxacillin; CX - Cefoxitin

MRSA confirmatory test

The phenotypic confirmation of Methicillin resistance *Staphylococcus* strains was assessed by using Oxacillin (1mcg) and Cefoxitin (30 μg) disc. Discs of Oxacillin (1 mcg) and Cefoxitin (30 μg) was placed separately on Muller Hinton agar (MHA) plate on which 0.5 McFarland of the test organism was swabbed. The organisms were considered as MRSA/MRSE producer if there was ≥5 mm increase in zone diameter of Oxacillin / Cefoxitin.

Genotypic confirmation of ESBL and MRSA/MRSE strains

Extraction of DNA from ESBL and MRSA/MRSE isolates was prepared essentially as described by Sambrook and Russell [10]. The primers used for identification of SCC *mecA* gene in *Staphylococcus aureus* and *Staphylococcus epidermidis* was forward primer mecA - 5′ TTGTCTGC CAGTTTCTCCTTG-3′ and reverse primer mecA – 5′ GAACAGCATATGAGATAGGCATC – 3′. The primers used for identification of $bla_{CTX\text{-}M}$ gene in *Pseudomonas aeruginosa* was forward primer CTX-M – 5'CGCTTTGC GATGTGCAG – 3′ and reverse primer CTX-M – 5'ACCGCGATATCGTTGGT- 3′.

PCR amplification

The reaction mixture consist of 50 μL of the master mix containing; Primer- Forward (5 μM/μL) - 5.0 μl, Primer - Reverse (5 μM/μL) - 5.0 μl, 10X Buffer - 5.0 μl, 2 mM dNTP Mix - 5.0 μl, *Taq* DNA polymerase (1 U/1 μL) - 2 μl, Template DNA - 5.0 μl and Nuclease free water - 23 μl. The PCR amplification cycle was carried out with an Eppendrof Master Cycle. The amplification cycle consists 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. To visualize the amplified PCR product 16 μl volume of the amplified products were subjected to electrophoresis in Tris - borate EDTA (1X TBE) pH 8.0 on a 0.8% agarose gel incorporating 0.01% ethidium bromide, 8 μl of the DNA ladder (1000 bp) were run in parallel to the samples in each gel. The amplified PCR products were visualized under a gel documentation unit (LARK Gel Documentation Imaging System, India).

Partial sequencing of the ESBL and MRSE strains

The strains which showed resistant to all the antibiotics were choosing for the partial sequencing. ESBL producing strain of *Pseudomonas aeruginosa* (APS01) and MRSE strain of *Staphylococcus epidermidis* (APS02) confirmed by molecular methods was subjected to partial sequencing by 16S rRNA sequencing. The universal bacterial primers were used for the

Table 2 Antibiotic susceptibility pattern of gram negative organisms from cases of diabetic foot infections

Name of the organisms	Total No of isolates	CFS	CTR	AT	CIP	PIT	AMC	CPZ	CAC	TCC	CPD	MRP	CPM	CFM	PI	AK	RIF
Pseudomonas aeruginosa	45	35(78)	35(78)	28 (62)	33(73)	44(98)	13(29)	35(78)	40(89)	20(44)	8(18)	37(82)	20 (44)	5 (11)	35 (78)	40(89)	8 (18)
Escherichia coli	6	6(100)	6(100)	3(50)	6(100)	6(100)	3(50)	6(100)	6(100)	6(100)	3(50)	6(100)	-	3(50)	6(100)	6(100)	6(100)
Enterococcus faecalis	5	3 (60)	3(60)	-	5 (100)	3 (60)	-	3 (60)	5 (100)	3 (60)	-	5 (100)	-	-	-	3 (60)	3 (60)
Klebsiella pneumoniae	4	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	-	4(100)	4(100)	4(100)	-
Salmonella paratypi	3	2(67)	-	-	2(67)	3(100)	-	-	-	-	-	-	-	-	2(67)	2(67)	-
Shigella sonnei	1	1(100)	-	-	1(100)	1(100)	-	1(100)	1(100)	1(100)	-	1(100)	-	-	-	1(100)	-
Proteus vulgaris	1	1(100)	-	-	1(100)	1(100)	1(100)	-	-	-	-	-	-	-	1(100)	-	-
Vibrio parahaemolyticus	1	1(100)	1(100)	1(100)	-	1(100)	1(100)	1(100)	1(100)	1(100)	-	1(100)	-	1(100)	1(100)	1(100)	1(100)
Pleisomonas shigelloides	1	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)

*CFS - Cefoperazone/Sulbactam; CTR - Ceftriaxone; AT - Aztreonam; CIP - Ciprofloxacin; PIT - Piperacillin/Tazobactam; AMC - Amoxyclav; CPZ - Cefoperazone; CAC - Ceftazidime/Clavulanic acid; TCC - Ticarcillin/Clavulanic acid; CPD - Cefpodoxime; MRP - Meropenem; CPM - Cefepime; CFM - Cefixime; PI - Piperacillin; AK - Amikacin; RIF - Rifampicin

partial sequencing of the ESBL and MRSE strains for genotypic confirmation. The forward primer was 5'GAGTTTGA TCCTGGCTCAG-3'and reverse primer – 5'ACGGCTAC CTTGTTACGACTT-3′. The PCR amplification cycle was carried out and the purified PCR fragments were sequence commercially at Yaazh Genomic Laboratory, Chennai, Tamilnadu using ABI 3730xl Analyzer.

Phylogenetic analysis

Using the CLUSTAL-W Multiple Sequence Alignment Program, the 16S rRNA sequences of the bacterial strains obtained in this study were aligned with sequences of related organisms obtained from Gen Bank. The phylogenetic analysis was performed with the help of Bio Edit and MEGA 5.1 software and a phylogenetic tree was constructed via the neighbor-joining method. To validate the reproducibility of the branching pattern, a bootstrap analysis was also further performed. The nucleotide sequences have been deposited in the NCBI Gene Bank and the accession numbers were received with relevant bacterial strains.

Results

This prospective study involving 160 patients presenting with diabetic foot infections at the outpatient department of the foot care hospitals, Salem, Tamilnadu, India. Out of 160 samples, 140 (87%) sample were culture positive and 20 (13%) were culture negative. A total of 215 organisms were isolated from 140 samples, 82% grew purely bacteria alone, 4% patients grew purely fungi alone and the remaining 14% patients grew a combination of bacterial and fungal organisms.

The bacteriological profile of the patients showed gram positive organisms more in number than the gram negative organisms. Monomicrobial infection (64%) was higher than the polymicrobial infections (22%) and no growth (14%). *Staphylococcus aureus* (35%) was the predominant one reported followed by *Staphylococcus saprophyticus* (12%), *Staphylococcus epidermidis* (4%), *Enterococcus fecalis* (3%), *Micrococcus luteus* (3%) and *Streptococcus pneumoniae* (1%). Among gram negative organisms *Pseudomonas aeruginosa* (25%) was the predominant organisms followed by *Escherichia coli* (3%), *Klebsiella pneumoniae* (2%), *Salmonella paratyphi* (2%), *Vibrio parahaemolyticus* (1%), *Shigella sonnei* (1%), *Pleisomonas shigelloides* (1%) and *Proteus vulgaris* (1%). The gram positive bacilli included *Bacillus subtilis* (6%) and *Listeria monocytogens* (1%). The antibacterial agents Amikacin, Tetracycline, Ciprofloxacin, Oxacillin, Cefoxitin and Vancomycin were found to be the most effective antibacterial agents against gram positive organisms (Table 1) while Meropenem, Piperacillin, Cefoperazone / Sulbactam, Piperacillin / Tazobactam, Ticarcillin / Clavulanic acid and Amikacin were found to be the most effective antibacterial agents against gram negative organisms isolated in this study (Table 2).

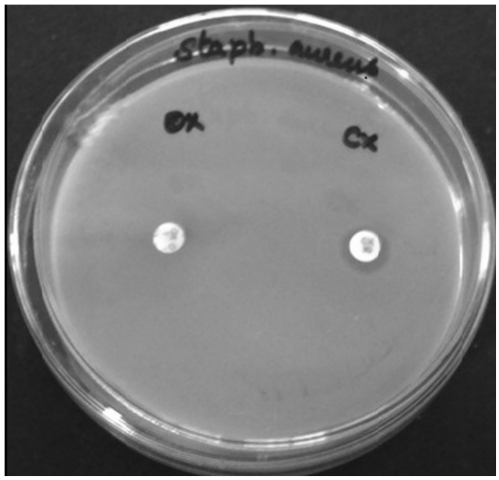

Fig. 1 *In - vitro* susceptibility pattern of MRSA strain

Out of the 91 isolates of *Staphylococcus* species screened for resistance, 4 isolates of Methicillin resistant *Staphylococcus aureus* (MRSA) (MRSA 4–2, MRSA 26, MRSA 91 and MRSA 101–1) (Fig. 1) and 1 isolates of Methicillin resistant *Staphylococcus epidermidis* (MRSE) (MRSE 82) were found to be MRSA and MRSE isolates as confirmed by phenotypic method. From the 67 gram negative isolates screened for resistance, 5 isolates of *Pseudomonas aeruginosa* (ESBL 33–2, ESBL 116, ESBL 119, ESBL 148–2 and ESBL 149) (Fig. 2) were found to be extended spectrum of β - lactamase (ESBL) producers as confirmed by this phenotypic method. Four strains of Methicillin

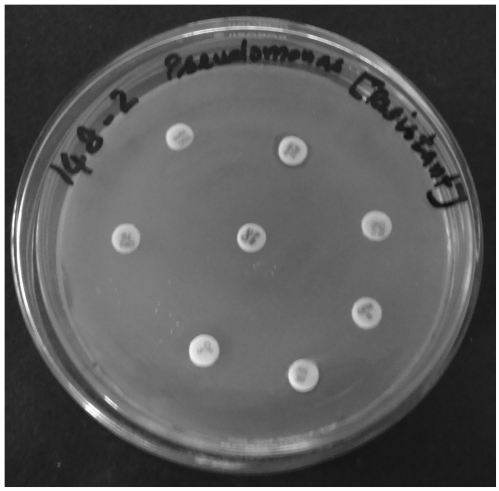

Fig. 2 *In - vitro* susceptibility pattern of *Pseudomonas aeruginosa* ESBL (148–2) strain

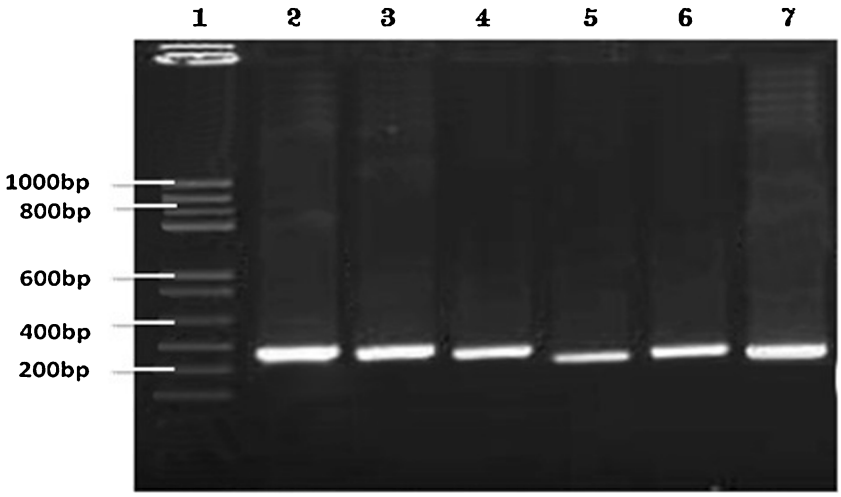

Fig. 3 Genotypic confirmation of MRSA/MRSE strains

Lane 1 - Marker; Lane 2 - Positive Control; Lane 3 to 6 - *Staphylococcus aureus* strain (MRSA); Lane 7 - *Staphylococcus epidermidis* strain (MRSE)

Resistant *Staphylococcus aureus* (MRSA) strains and one strain of Methicillin Resistant *Staphylococcus epidermidis* (MRSE) strain were subjected to genotypic confirmation for SCC *mecA* gene. The PCR amplified product showed DNA fragments of 220 bp for *mecA* gene in the four isolates of *Staphylococcus aureus* (MRSA 4–2, MRSA 26, MRSA 91, MRSA 101–1) and one isolate of *Staphylococcus epidermidis* (MRSE 82) confirming the presence of *mecA* genes in these strains conferring resistance to the isolates (Fig. 3).

Five strains of ESBL producing *Pseudomonas aeruginosa* which were confirmed by phenotypic method were further subjected to genotypic confirmation for $bla_{CTX\text{-}M}$ gene. PCR amplified product showed DNA fragments of 130 bp for $bla_{CTX\text{-}M}$ gene of *Pseudomonas aeruginosa* (ESBL 33–2, ESBL 116, ESBL 119, ESBL 148–2 and ESBL 149) confirming the presence of $bla_{CTX\text{-}M}$ genes in these strains conferring resistance to the isolates (Fig. 4). The genome of ESBL *Pseudomonas aeruginosa* (ESBL 148–2) and Methicillin resistant *Staphylococcus epidermidis* (MRSE 82) strains were subjected to 16S r RNA sequencing. The results of the phylogenetic analysis confirmed that the bacterial strain *Pseudomonas aeruginosa* designated as APS01 was found to be closely related to *Pseudomonas sp.* CEBP1 (Fig. 5) and *Staphylococcus epidermidis* designated as APS02 was closely related to *Staphylococcus epidermidis* 2D (Fig. 6). The strains of *Pseudomonas aeruginosa* (APS01) and *Staphylococcus epidermidis* (APS02) sequences were deposited in the GenBank database with the accession number as KJ918742 and KM502278 respectively.

Discussion

The diabetic foot infection is part of the main causes of morbidity which progress to lower limb amputation. Despite the fact that there are many studies with regard to the clinical profile and

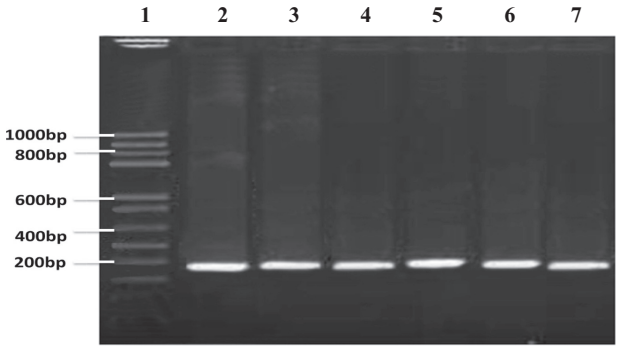

Fig. 4 Genotypic confirmation of ESBL strains

Lane 1- Marker; Lane 2 - Positive control; Lane 3 to 7- *Pseudomonas aeruginosa* strains (ESBL)

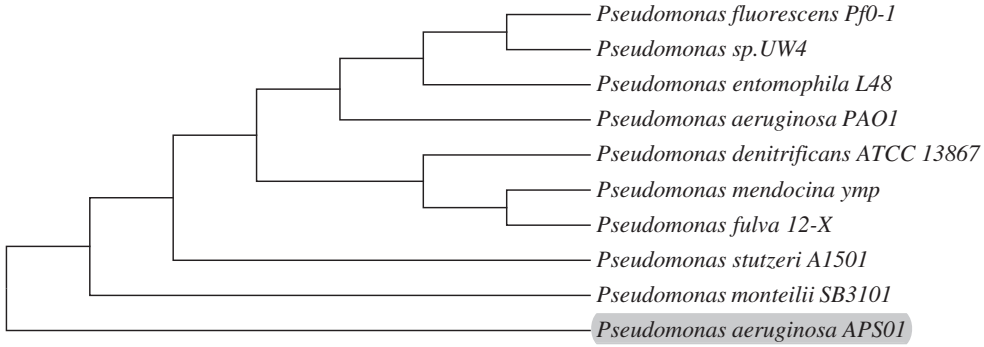

Figure 5 Phylogenetic analysis of *Pseudomonas aeruginosa* strain APS01

microbiology of the foot infections, but the magnitude of the problem often remains the same [11]. This study evaluated the molecular characterization of resistant genes in bacterial population of diabetic foot ulcers. In this study, there were a higher percentage of mono microbial infections (64%) a finding similar to the study of Dhanasekaran et al. [12] and Tiwari et al. [13] which reported 43.5% of mono microbial infections. The bacteriological profile of the patients showed gram positive organisms occur in greater number than gram negative organisms. The observation was the same as the one reported by Abdulrazak et al. [14] which reported gram positive organisms formed almost >60% of the clinical isolate because of the mild and moderate form of the disease. The predominant organism was *Staphylococcus aureus* (35%) followed by *Pseudomonas aeruginosa* (25%). The findings of this study were similar to Sharma et al. [15] which reported higher percentage of *Staphylococcus aureus* namely 38.4%. The antimicrobial susceptibility pattern was similar to the findings of Rajalakshmi and Amsaveni [16] which also showed Vancomycin as the most effective antibiotic for gram positive organisms and Imipenem, Meropenem, Amikacin, Piperacillin / Tazobactam as the most effective antimicrobial agents for gram negative organism.

The incidence of multi-drug resistant organisms, namely Methicillin resistant *Staphylococcus aureus* and Extended spectrum beta lactamase are a serious concern and threatening the outcome of therapy. The presence of Methicillin resistant *S. aureus* (MRSA), Methicillin resistant *Staphylococcus epidermidis* (MRSE) and Extended spectrum of beta Lactamases (ESBL) was confirmed by phenotypic method. These observations were comparable to Lipsky [17] and Citron et al. [18] which reported Oxacillin resistant *Staphylococcus aureus* in diabetic foot infection. This study also correlated with the study of Zubair et al. [19] in which 68.5% isolates were ESBL producers isolated from diabetic foot ulcer infection in a North Indian tertiary care hospital, New Delhi, India and later on in year of 2012 Zubair et al. [7] demonstrated that 74% of gram negative diabetic foot isolates were positive for ESBL by the disc diffusion method. Thus the study suggested that the mild infection was monomicrobial in etiology, as the causative organisms showed 100% sensitivity to Oxacillin. If the infection was severe and involving deep tissue, Ceftazidime, Imipenem, Meropenem and Ciprofloxacin will be more appropriate with their sensitivities reaching 98% to 100%.

In genotypic detection, this study confirmed the presence of *mecA* gene in the MRSA/MRSE isolates. The findings of the study was correlated to Zhang et al. [20] which screened SCC *mec* type in a Canadian Methicillin-resistant *Staphylococcus aureus* (MRSA) by multiplex and conventional PCR methods. In a yet another study by Miragaia et al. [21] which reported that Methicillin resistant *S. epidermidis* isolates exhibiting *mecA* gene conferring resistant to all classes of antibiotics. Similar observations were

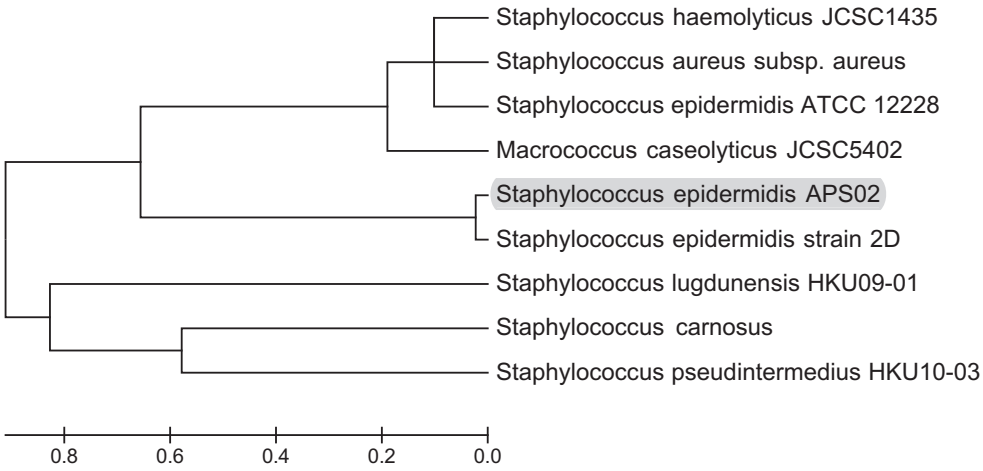

Figure 6 Phylogenetic analysis of *Staphylococcus epidermidis* strain APS02

made by Oliveira and Lencastre [22] in which Methicillin resistant *Staphylococcus epidermidis* strains appeared 57.9% of *mecA* gene. The CTX-M enzymes predominate among the ESBLs of community strains [23]. The CTX-M provides the pathogens with an additional resistance mechanism giving rise to potentially serious clinical implications. This study revealed the presence of $bla_{CTX\text{-}M}$ genes was correlated Naiemi et al. [24] which first reported the presence of the $bla_{CTX\text{-}M\text{-}1}$, $bla_{SHV\text{-}1}$ and $bla_{TEM\text{-}116}$ genes in the *P. aeruginosa* strains. Polotto et al. [25] detected the $bla_{CTX\text{-}M\text{-}2}$ in eleven isolates (19.6%) and the study by one of the author Farshadzadeh et al. [26] found the coexistence of $bla_{CTX\text{-}M}$ in sixty six isolates of *P. aeruginosa* from infectious wounds.

The strains *Pseudomonas aeruginosa* (ESBL 148–2) and *Staphylococcus epidermidis* (MRSE 82) which were resistant to all the antibiotics were subjected to 16Sr RNA sequencing. A similar approach of molecular tools by using the 16S rRNA gene was done by Relman et al. [27] which confirmed *P.aeruginosa* by sequence analysis of the 16S rRNA gene. The study of *Staphylococcus epidermidis* was correlated with the study of Sanches et al. [28] found that the origin of diversity in *S. epidermidis* contains sequences coded for biofilm production and antibiotic resistance. Otto [29] stated that *S.epidermidis* harbors virulence determinants, but antibiotic resistance contributes to the persistence of clinical infection. Thus, this study confirmed the presence of multidrug resistant strains in diabetic foot infections especially in Southern most part of India. While the foot infected patients undergoes several factors such as inappropriate antibiotic treatment, the chronic nature of the wound and recurrent hospital admissions leads to the presence of multidrug resistant microorganisms in the ulcer [30]. So the initial management of foot infection comprises empirical antimicrobial treatment based on the susceptibility data. The eradication of these resistant strains was done only by therapy directed at knew causative organisms could significantly improve the outcome and reduce the infection related morbidities.

Conclusion

Antibiotic resistance is only an alarming and growing concern. The degree of microbial load associated with foot infection and their potential provides for dissemination of resistant organisms. The proper selection of antibiotic for a specific pathogen and therapeutic dose can save amputation from infections. So the early recognition of infections and prompt initiation of appropriate antibiotic therapy is essential factors for successful therapeutical outcome.

Compliance with ethical standards

Funding None.

Conflict of interest P. Sugandhi declares that she has no conflict of interest. D. Arvind Prasanth declares that he has no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):108–114
DOI 10.1007/s13410-017-0556-3

ORIGINAL ARTICLE

An investigation of the foot ankle joint mobility, muscle strength, and foot structure in adolescent with type 1 diabetes

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Received: 9 September 2016 / Accepted: 21 March 2017 / Published online: 30 March 2017

Abstract Muscle strength and joint mobility were decreased with type 1 diabetes mellitus (T1DM). However, the literature is limited about foot muscle strength, joint mobility, and structure in adolescent aged 13–17 with T1DM. The purpose of this study was to compare foot structure, muscle strength, and joint mobility of adolescents aged between 13 and 17 with T1DM to those of healthy adolescents. Cross-sectional study design including adolescents with T1DM aged 13–17 years, and healthy adolescents was used in the study. The range of motion (ROM) was measured by using a digital goniometer, and muscle strength was evaluated by using handheld-dynamometry. Footprint was used for foot structure. Assessments were performed by using the digital images, and Clarke's angle (CA), Staheli Arch index (SAI), and Chippaux-Smirak index (CSI) were calculated by using a scientific image-analysis program, ImageJ. Forty-one T1DM and 28 healthy adolescents were included with a mean age of 15.29 ± 1.55 and 15.04 ± 1.42, respectively. The T1DM group had significantly lower dorsiflexion, inversion and eversion ROM, and lower tibialis anterior and gastrocnemius muscle test ($p < 0.05$, for all) compared with the control group. Statistically, significant differences were found in the right SAI and CSI between groups ($p < 0.05$), whereas no difference was found in CA ($p > 0.05$). Adolescents with T1DM have lower ankle and foot joint mobility and muscle strength and altered foot structure compared to their healthy contemporaries. This indicates that early screening of muscle strength and foot structure are important to determine and avoid various risks such as foot deformities, gait deviations, and ulcer.

Keywords Type 1 diabetes mellitus · Footprint · Adolescent · Muscle strength

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Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease defined as destruction of the insulin producing β-cells of the islets of Langerhans in pancreas [1, 2]. It is estimated that there are approximately 500,000 adolescents with T1DM aged under 15 worldwide, and T1DM is defined as the second most common chronic illness among school-aged children [3, 4].

There are many complications of diabetes caused by hyperglycemia and hypoinsulinemia, highly associated in retrospective studies with poor diabetes control [2, 5]. Musculoskeletal conditions affecting joints, soft tissues, nerves, muscles, and tendons are among these complications [6]. Motor dysfunction is a late result of diabetic polyneuropathy and leads to muscle weakness related to insufficient reinnervation, which is caused by loss of motor axons [7]. There are studies having shown the reduction of lower extremity muscle strength, atrophy of dorsal and plantar flexors, reduced intrinsic foot muscle volume in diabetes [8–10].

The atrophy of foot muscles is known to be a cause of altered foot structure [11]. Weakness of tibialis posterior muscle is associated with acquired pes planus because of

insufficient medial longitudinal arch support [12]. Atrophy of the small muscles is thought to lead to development of pes cavus, pes equinus hammer toes, claw toes, hallux valgus, and prominent metatarsal heads [13]. Andreassen et al. [7] reported that muscular atrophy occurs early in the feet and progresses in the lower legs in T1DM patients. This progression can lead muscle imbalance that causes weakness of the intrinsic muscles with relatively strong long toe flexor, extensor, and ankle muscles, therefore, toe abnormalities like hammertoe, claw toe, and pes cavus occur [14]. Foot deformities are associated with high plantar pressure areas, foot ulceration, and finally lower limb amputations [15]. In addition, Toth et al. [16] found that functional motor unit was reduced before neuropathy findings in adolescents T1DM. The ISPAD Guideline for Diabetes in Childhood and Adolescence recommend annual foot screening to assess feet problems from age of 11 within 2 years of diabetes duration in T1DM [17]. Therefore, early screening of joint mobility, muscle strength, and foot structure are important to prevent children with T1DM from deformities.

To our knowledge, the muscle strength and joint mobility decrease with T1DM in adults [10, 8, 7, 11, 18, 19]. The recent study showed that functional and structural foot problems are frequent in children with T1DM [20].However, the literature is limited about foot muscle strength, joint mobility, and structure in adolescent aged 13–17 with T1DM. This study aimed to compare foot structure, muscle strength, and joint mobility of adolescents aged between 13 and 17 with T1DM to those of healthy adolescents.

Material and methods

Participants

This is a cross-sectional study carried out from January 2015 to September 2015. The study was jointly conducted by the Bezmialem Vakif University Faculty of Medicine, Department of Pediatrics Endocrinology and the …….Istanbul University, Faculty of Health Sciences, Division of Physical Therapy and Rehabilitation. Inclusion criteria were age 13–17 at recruitment and diabetes duration greater than 2 years. Exclusion criteria were (1) lower extremity injuries within 6 months, (2) any other diseases affecting foot structure such as joint pathology, congenital vertical talus, cerebral palsy, or other musculoskeletal and neurological disorders, (3) pain associated with foot, and (4) diabetes diagnosis known to be non-type 1. Healthy participants with no current or past medical diagnoses or injuries affecting foot structure were included in the control group.

Ethical approval for this study was obtained from the Human Research Ethics Committee of Bakirkoy Prof. Dr. Mazhar Osman Research and Training Hospital for Psychiatry, Neurology and Neurosurgery (IRB: …….2015/474). All participants and their parents were informed about the study and the informed consent was obtained.

We performed a power analysis to determine sample size at the beginning of the study. Sample size was calculated by Raosoft, Inc. With a power of 90%, an error >5%, and 3% for incidence of T1DM adolescent, the minimal sample size was estimated at 32 participants to detect a statistically significant difference between the T1DM group and control group. Allowing for a conservative dropout rate, we recruited 42 adolescents with T1DM into the study.

Of the patients, aged 13–17 who were examined in the pediatrics endocrinology clinic within the study period, the first 42 and their parents were called by phone, and they were willing to participate in the study. They were invited to an interview in ……..Istanbul University. One participant was excluded from the study as they met exclusion criteria. Therefore, 41 adolescents (91.1%) (18 boys and 23 girls) and their parents were included.

Control group was selected from healthy adolescents in a secondary and high school near the hospital. Control group was formed from students aged 13–17 who met inclusion criteria and who came to school on selection day. Thirty healthy controls were included as control group. Two healthy controls were not willing to participate. Therefore, 28 healthy controls (93.3%) participated in the study.

Demographic data form was fulfilled by a physical therapist. Participants' weight and height were measured, and body mass index was calculated as weight/height^2. Clinical features such as disease duration and HbA1c were assessed by an endocrinologist. HbA1c levels represented the measurements in previous 1 month.

Outcome measures

Outcomes measures included range of motion (ROM), muscle strength, and footprint analysis. The assessment was performed by the same physiotherapist at a university research clinic, secondary and high school. Data analysis were made by another physiotherapist.

Range of motion

The active ROM of each participant, including ankle dorsiflexion and plantarflexion and foot inversion and eversion, were measured as described by Clarkson [18] by using universal goniometer. Ankle dorsiflexion and plantarflexion ROM measurements were performed in a long sitting position with a pillow under the knee. The foot was in the neutral position "0" degree. The axis was placed approximately 1.5 cm inferior to the lateral malleolus. The stationary arm of the goniometer was placed parallel to the longitudinal axis of the fibula, and the moving arm was placed parallel to the longitudinal axis of the fifth metatarsal. Next, the ankle was

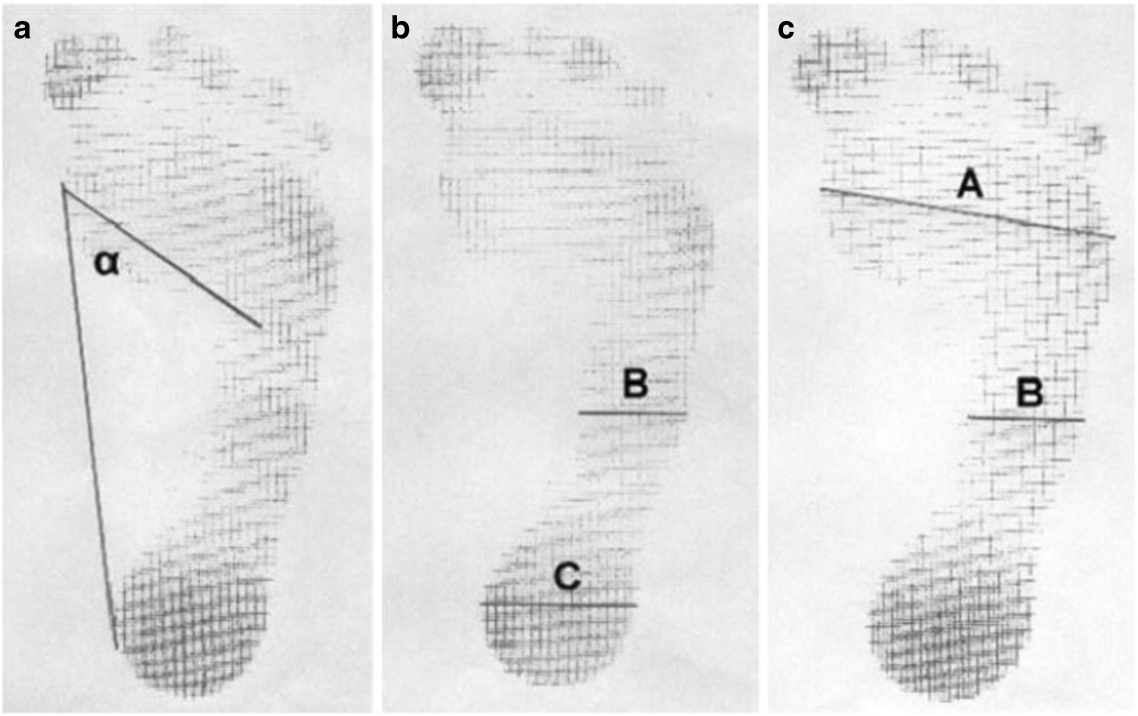

Fig. 1 Footprint analysis

flexed with the dorsal aspect of the foot approximating the anterior aspect of the lower leg for dorsiflexion ROM. The ankle was extended to the limit of motion for plantarflexion ROM. The ROM was measured with the universal goniometer, while the participants were doing active motion. Foot inversion and eversion ROM measurements were performed in a supine lying position. The ankle was in the neutral position. A piece of paper was placed under the foot. A flat-surfaced object (plexiglass) was placed against the full sole of the foot. A line was drawn parallel to the plexiglass. The foot was placed in inversion to the limit of motion. The plexiglass was positioned against the full sole of the foot, and a line was drawn parallel to the plexiglass. The process was repeated for eversion to the limit of motion. The goniometer was placed on the line graphics to obtain a measure of the arc of the movement. In this study, three repetitions were performed in each direction, and the average value was recorded for analysis.

Muscle strength

The strength of tibialis anterior and gastrocnemius muscles of both side were measured with a handheld dynamometer (HHD) (kg/Newton) "Nicholas Manual Muscle Tester" (The Lafayette Instrument Company, Lafayette, Indiana, model 01160) [19]. This dynamometer allowed a measurement of muscle strength from 0.0 up to 199.9 kg, with 0.1 kg precision. The test participants were allowed to make a one-time practice in order to learn the test procedure [21]. The test participants were asked to contract his/her muscle forcibly, while a resistive force was applied with the dynamometer in the opposite direction of the intended movement. During the test, the device recorded the maximal isometric strength of the muscle in kg/Newton. Tibialis posterior and peroneus longus muscle strength were measured by manual muscle test (MMT). Traditional MMT grades were used [18]. Numerals (0–5) were used to indicate grades of muscle strength. The full available ROM against gravity and against maximal resistance was graded 5. None of the available ROM gravity eliminated was graded 0. Each muscle was assessed three times, and mean value was calculated. The reliability of the MMT in children has been previously reported (interclass correlation coefficient > 0.8) [22].

Footprint analyses

Static footprints of patients were collected with a Harris-Beath mat for each foot on standing weight-bearing position and then they were converted to digital images with a scanner. Assessments were performed by using the digital images, and Clarke's angle (CA), Staheli Arch index (SAI), and Chippaux-Smirak index (CSI) were calculated by using a scientific image-analysis program, ImageJ. The angle and length measurement of the program were used for the measurements (NIH Image to ImageJ, history paper).

CA, explored by Clarke [23–25], is the angle between a first medial tangential line that connects the medial edges of the first metatarsal head and the heel, and a second line

Table 1 Demographic and clinical features of T1DM and healthy groups

Demographic	T1DM group $n = 41$ (min–max)	Healthy group $n = 28$ (min–max)	p value
Age (years) (mean ± SD)	15.29 ± 1.55 (13–17)	15.04 ± 1.42	0.433
BMI (kg/m^2)	20.91 ± 3.37 (15.53–30.72)	20.87 ± 3.31 (15.23–29.13)	0.920
Sex (M/F)	18/23	16/12	0.281
Disease duration (years)	2.83 ± 0.83	–	–
HbA1c	7.98 ± 1.46 (4.7–11.5)		

T1DM Type 1 diabetes mellitus, *SD* standard deviation, *BMI* body mass index, *F* female, *M* male, *HbA1c* hemoglobin A1c

aligning the first metatarsal and the MLA's first contact point, intersecting at the first metatarsal head. Higher values determine a trend toward a cavus foot, and lower values a trend toward flattening or pronation (Fig. 1a). A reliability coefficient of 0.971 was reported for CA [23, 24]. The SAI, studied by Staheli et al. [26, 25, 24], was stated as an indicator of foot arch development, and it was defined as the ratio of the length of narrowest point on the foot arch (line B) to maximum width of the heel area (line C) (B/C × 100%) (Fig. 1b). The CSI was defined as the ratio of the length of line B, to the maximum width at the metatarsals (line A) (B/A × 100%), and it was calculated by Forriol and Pascual [27, 25] to describe arch development (Fig. 1c). Higher values determine a flattening or pronation trend, and lower values determine a trend toward a cavus foot in SAI and CSI measurement. Queen et al. [28] found good intrarater and interrater reliability for SAI (0.914 and 0.962, respectively) and CSI (0.913 and 0.960, respectively). Forriol and Pascual [27] classified the foot structure according to CA and CSI. CA between 0° and 29.9° shows a flatfoot, between 30° and 34.9° shows a foot with a low medial longitudinal arch, between 35° and 42° shows an intermediate foot, and above 42° shows a normal foot; CSI values over 45%, from 40 to 44.9%, from 30 to 39.9%, from 0.1 to 29.9%, and 0 demonstrate flat foot, low medial longitudinal arch, intermediate foot, normal foot, and cavus foot respectively according to this classification. Echarri and Forriol [29] classified the foot structure by considering the SAI as a fallen medial longitudinal arch for values above 90%, a descending medial longitudinal arch for values from 60 to 89%, a normal foot for values from 30 to 59%, and a high medial longitudinal arch for values from 0 to 29%.

Statistical analysis

Data was evaluated by using the Statistical Package for Social Science 21.0 program for Windows and by analyzing descriptive statistics (frequency, mean, and standard deviation). Before the statistical analysis, Kolmogorov–Smirnov test was used to test normal distribution of data. All continuous variables were not normally distributed. Mann Whitney U test was used to compare age, BMI, range of motions, muscle test, and footprint analysis between T1DM and control groups. Chi-square test was used to compare sex ratio between two groups. P values less than <0.05 were considered as statistically significant.

Results

Analysis between the two groups indicated no significant differences in demographic background. Group characteristics were presented in Table 1. The mean age was 15.29 ± 1.55 (range, 13–17) in T1DM group and 15.04 ± 1.42 (range, 13–17) in control group. The mean disease duration at the time of enrollment was 2.83 ± 0.83 (min–max: 2–5 years).

The comparison results of range of motion, muscle strength, and footprint analysis were shown in Table 2. The T1DM group had significantly reduced dorsiflexion, inversion and eversion range of motion, and reduced tibialis anterior and gastrocnemius muscle test ($p < 0.05$, for all) compared with the control group. Statistically, significant differences were found in the right SAI and CSI between groups ($p < 0.05$), whereas no difference was found in CA ($p > 0.05$) (Table 2).

Discussion

The purpose of this study was to determine the differences of foot and ankle muscle strength, foot structure, and joint mobility in adolescent with T1DM. Compared to their healthy peers, adolescents with T1DM had a significantly lower ankle and foot range of motion, a lower ankle muscle strength, and a tendency to high medial longitudinal arch.

Table 2 The comparison between the two groups

Parameters		T1DM group $n = 41$	Healthy group $n = 28$	p value
Range of motion (°)				
Dorsiflexion	R	16.09 ± 3.67	18.25 ± 2.64	***0.008***
	L	15.69 ± 3.81	18.22 ± 2.63	***0.004***
Plantarflexion	R	43.35 ± 2.95	44.54 ± 1.34	0.092
	L	43.34 ± 2.77	44.54 ± 1.23	0.051
Inversion	R	33.40 ± 2.60	35.0 ± 0	***0.002***
	L	33.47 ± 2.41	35.0 ± 0	***0.002***
Eversion	R	13.53 ± 2.19	15.0 ± 0	***0.001***
	L	13.51 ± 2.21	15.0 ± 0	***0.001***
Muscle strength				
Tibialis anterior (kg/Newton)	R	8.46 ± 2.97	12.83 ± 3.17	***<0.001***
	L	8.35 ± 2.51	12.22 ± 3.11	***<0.001***
Gastrocnemius (kg/Newton)	R	10.99 ± 3.96	17.87 ± 3.66	***<0.001***
	L	11.06 ± 3.80	16.63 ± 4.48	***<0.001***
Tibialis posterior	R	4.98 ± 0.15	5.0 ± 0	0.403
	L	4.98 ± 0.15	5.0 ± 0	0.233
Peroneus longus	R	4.96 ± 0.26	5.0 ± 0	0.403
	L	4.95 ± 0.22	5.0 ± 0	0.403
Footprint analysis				
CA (°)	R	42.64 ± 10.03	44.06 ± 9.74	0.521
	L	43.21 ± 10.24	42.44 ± 11.06	0.827
SAI (%)	R	55.53 ± 19.01	63.03 ± 19.84	***0.041***
	L	55.87 ± 18.87	63.91 ± 17.10	0.079
CSI (%)	R	30.82 ± 10.95	35.42 ± 12.61	***0.049***
	L	31.17 ± 11.19	35.75 ± 11.08	0.071

Bold and italic number indicates a statistically significance between groups

T1DM Type 1 diabetes mellitus, *CA* Clarke's angle, *SAI* Staheli arch index, *CSI* Chippaux-Smirak index

Our results showed that foot dorsiflexion, inversion, and eversion ranges decrease in adolescent with T1DM. It is known that limited ankle, subtalar, and first metatarsophalangeal joints are common in T1DM in long-term, and this feature is specified as an early complication of pediatric diabetes [30]. It has been reported that joint limitation of the ankle and foot leads foot deformities, gait abnormalities, and increase plantar pressures [31, 13]. Francia et al. [31] indicated that evaluation of the ankle joint mobilization is important in determining the ulcer risk of the foot. Nagesh and Kalra [32] specified that limited joint mobilization is reversible in early stages but becomes irreversible in time. Our present findings considered together with those results of the literature show ankle and foot joint mobility have to be evaluated and prevented to avoid further foot problems in adolescents with T1DM from early stages.

We found that tibialis anterior and gastrocnemius muscles are weaker in adolescents with T1DM from the healthy controls. Although muscle weakness is known to be a late result of diabetic neuropathy, Greenman et al. [33] indicated that atrophy of foot small muscles can be found before clinical neuropathy diagnosis. Toth et al. [16] pointed that motor unit loss begins in early stages before clinical signs of peripheral neuropathy in adolescent with T1DM. Early detection of ankle and foot muscle strength can prevent foot deformities, gait alterations, and increased plantar pressures in adolescent with T1DM.

This study indicated SAI and CSI values were lower in T1DM group which means a trend toward pes cavus [27, 26]. Barnett et al. [34] showed a significant increase in foot pathologies and biomechanical abnormalities in diabetic children compared to healthy ones. In line with Barnett et al. [34], and Wever et al. [20] found that a high percentage of children and adolescents with T1DM had functional and structural foot abnormalities. Furthermore, muscle weakness progresses from distal to proximal in consequence of motor neuropathy leading to atrophy in the intrinsic muscles before the extrinsic muscles [35, 36]. This condition is known to cause muscle imbalance which results in hammer toes, claw toes, and pes cavus [35]. Our results which show a foot structure deviation toward pes cavus are consistent with this knowledge. A recent study showed that children aged of 8 to 15 years old are prone to a

flatter foot posture [37]. Our study shows that children with T1DM have different foot structure from healthy children. On the other hand, the foot type of the adolescents were not classified according to footprint evaluations in the present study, because the mean values of CA, SAI, and CSI were found as normal medial longitudinal arch for both healthy and T1DM groups by considering to the classification that defined above. Therefore, we aimed to demonstrate whether there is a tendency to deviate from normal foot structure based on the studies that investigated the foot structure by using CA, SAI, and CSI in different populations [38, 39]. Chen et al. [25] demonstrated cutoff points as CA ≤ 14.04°, CSI > 62.70%, and SAI > 107.42% for flatfoot in preschool-aged children, and Pita-Fernandez et al. [40] determined cutoff points CA ≤30.5°, CSI ≥ 45.75%, and SAI ≥0.825% for flatfoot in adults aged 40 years and older. Villarroya et al. [41] indicated morphological flatfoot in adolescents with obesity aged between 9 and 16.5 years, and their study showed a decrease of CA and an increase of CSI in obese adolescents compared to healthy ones. However, there was no study that investigated the foot type by using footprint analysis in T1DM children or adolescents.

This study demonstrates that reduced ankle and foot joint mobilization, muscle strength and altered foot structure arise in children with T1DM from early stages. As these problems are associated with foot deformities, gait abnormalities, increased plantar pressures, and foot ulcer, it is important to evaluate, follow, and avoid ankle and foot joints, muscles, and structure in children with T1DM. Allan et al. [13] presented that the literature is limited to support motor neuropathy, muscle weakness, and imbalance leads foot deformities. Further research is needed to find out these associations. Francia et al. [42] obtained that exercise therapy improves joint mobility, muscle performance, and gait speed in diabetes. Singla et al. [43] reported that musculoskeletal diseases affect the quality of life in diabetic patients, and early identification can reduce long-term morbidity. These findings show the importance of early evaluation, detection, and treatment of foot problems in adolescents with T1DM.

There is a need for further evidence to evaluate the foot muscle strength and foot structure with more objective methods and their long-term follow-up. The present study did not use more objective assessment methods and did not evaluate the development of these foot problems in time. The results of both the T1DM and control groups were measured by the same physiotherapist; therefore, the study was not blind. Also, more accurate results about the muscle strength, joint mobility, and foot structure in adolescents with T1DM can be obtained if a larger number of subjects were included in this study. Besides glycemic control, background of physiological and socioeconomic status might influence the outcomes of this study. These can be considered as limitations of the study.

In conclusion, adolescents with T1DM have reduced ankle and foot joint mobility and muscle strength and altered foot structure compared to their healthy contemporaries. This indicates evaluation and prevention of these foot problems are important to determine and avoid various risks such as foot deformities, gait deviations, and ulcer. In consistent with ISPAD guideline [17], regular control of ankle and foot joint mobility, muscle strength and foot structure are important in this population to protect further problems. Further research is needed to evaluate the prognosis and relationships of foot problems and effects of the different treatment methods on these problems in adolescents with T1DM.

Acknowledgments We thank all the participants involved in the survey. The help of ……..Omer Inan from …….Bandirma Onyedi Eylul University for the English review of the manuscript is very much acknowledged.

Compliance with ethical standards

Funding This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

There is no financial support or other benefits from commercial sources for the work.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):115–123
DOI 10.1007/s13410-017-0554-5

ORIGINAL ARTICLE

Factors contributing to effective referral systems for patients with non-communicable disease: evidence-based practice

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Received: 23 August 2016 / Accepted: 21 March 2017 / Published online: 29 March 2017

Abstract Referrals are an important part of the management of patients, particularly those with a chronic non-communicable disease (NCD), as it ensures the patient has continuous care. The main objective of this research is to develop a tool for assessing referral systems between primary and secondary care. The research question addressed in this paper is 'what constitutes an effective evidence-based practice (EBP) referral system?' Two steps were undertaken in this paper. In the first step, a question was formulated using a modified version of the PICOT (populations, interventions, comparison, outcome, and time) format, where only the PIO (patient, intervention and outcome) steps were used. The research question was used to search databases for systematic reviews on the factors contributing to effective referral systems for patients between primary and secondary care. The final part of the first step was to extract and synthesise data. The second step was to design an assessment tool that was able to assess referral studies in terms of the quality of the referral system. Three systematic reviews were included in this study, after being examined against the inclusion and exclusion criteria. The criteria for EBP referral systems consist of ten factors. The ten factors identified that an EBP referral system should be safe, timely, effective, efficient, patient-centred, and equitable; a referral letter should be structured; referral letter guidelines should be disseminated; a central computerised system should be used, and inclusion criteria of referred patients should be given in a referral letter. This ten-factor assessment tool will be used to assess referral systems as it is based on EBP from high quality studies and organisations. Using this tool could help researchers of referral systems to measure the problems faced in the referral system in their practice and address these problems, based on the ten factors.

Keywords Evidence-based practice (EBP) · Referral · Type 2 diabetes · Systematic review · Primary care · Secondary care · NCD · Chronic disease

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Introduction

A referral system is a crucial component in the management of many diseases. More significant is its use in the management of continuous care between different levels of care. Referral is defined as a process by which a healthcare professional manages a clinical condition by referring patients to other healthcare facilities due to insufficient resources (drugs, equipment, and skills) to seek assistance from a better or differently resourced healthcare facility at either the same or a higher level [1]. A referral is a two-way process that organises the patient flow from a lower to a higher level of care and vice versa. Every country has different healthcare systems and referrals that are used in different ways. Globally, referral systems face many challenges and obstacles because of the high demand for healthcare services. In many countries, a referral system does not follow the WHO guidelines [2–4]. Globally, non-communicable diseases (NCD), including heart

disease, stroke, cancer, persistent respiratory diseases, and diabetes, are some of the major causes of mortality [5]. Patients suffering from these diseases need continuous care, which is usually managed between more than one level of care. One way of coordinating care and managing these conditions is through the use of referrals, regardless as to whether the patient is being referred to a higher or lower level of care. A referral plays an important role in the management of NCD chronic conditions. According to several studies that examined chronic disease management, the provision of cooperative care for patients by primary care clinicians and specialists resulted in better health outcomes in comparison with each level acting alone [6–8].

Research studies have indicated that evidence-based practice (EBP) leads to high quality healthcare systems, better patient outcomes, lower cost, and greater satisfaction than traditional research methods [9, 10]. EBP is defined as an approach to solve healthcare problems and enhance decision making in which a researcher conducts a search for the latest and best evidence which includes clinical expertise and assessment and patient preference principles within a healthcare context [11]. Therefore, the research question posed in this study is which referral systems/interventions are effective in facilitating the better management of patients between primary and secondary care? The main objective of this paper is to develop a tool for assessing referral systems between primary and secondary care.

Method

Research design and methods

As shown in Fig. 1, two steps were undertaken in the research for this paper. In the first step, a research question was formulated using a modified version of PICOT (populations, interventions, comparison, outcome, and time), where only the PIO steps were used. The research question was used to search databases for systematic reviews on the factors contributing to effective referral systems for patients between primary and secondary care as well as other searches on the quality of referrals in some organisations. The included systematic reviews had previously been assessed by the Canadian Agency for Drugs and Technology in Health (CADTH) as having medium to high quality using the AMSTAR checklist [12]. The final part of the first step was to extract and synthesise the data. The second step was to develop a data collection tool to assess the selected studies on referral systems. This included a literature search on the quality of referrals and the WHO guidelines relating to referrals.

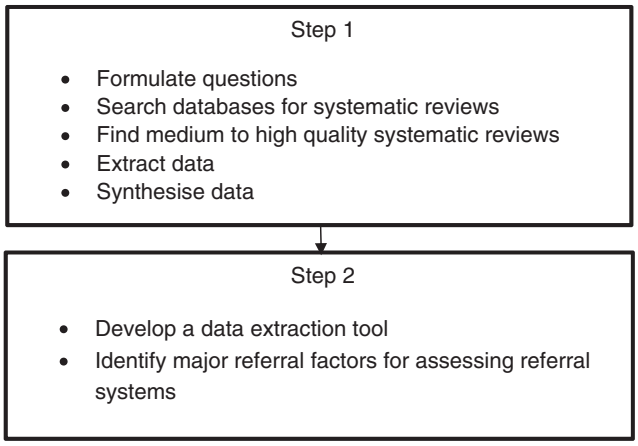

Fig. 1 Overview of the two-step approach used in this study

Formulation of answerable questions

The PICOT model is a tool that assists researchers organise and focus questions into a searchable query. The PICOT elements help identify search terms and concepts to use in searching the literature. In this paper, it was modified to PIO, as follows:

P = Patient problem, people/populations: How would you describe a group of patients similar to you? What are the most important characteristics of the patient?

I = Interventions, issues, prognostic factor, exposure: What is the main intervention you are considering? What do you want to do with or for these patients?

O = Outcome: What are you trying to accomplish, measure, improve or affect?

This modified format of the PIO was the focus of this paper, with focussed answerable questions as listed in Table 1. The research question for this paper is which referral systems are effective in introducing better management for patients between primary and secondary health care? We used type 2 diabetes as an example of a chronic disease.

Table 1 Research question

PIO model	
Population (P)	Patients with type 2 diabetes and other patients who need a referral
Intervention (I)	• Structured referral letter • Education about referral • Computerised systems • Dissemination of guidelines to primary and secondary health care services
Outcome (O)	Better health status, improved quality of life, reduced morbidity and mortality, and decreased complications

Literature search for identifying systematic reviews

A systematic literature search was conducted to identify reviews which were relevant to referral systems between primary and secondary health care for patients with type 2 diabetes. The search focused on papers which were effective in improving patients' health status and quality of life and were effective in reducing morbidity and mortality. The search strategy was then repeated in order to identify primary studies.

In order to ensure an unbiased systematic approach to database searching and the inclusion of relevant reviews, expert advice was sought. By utilising search terms in a manner recommended by Cochrane Systematic Reviews [13] and also based on the health-evidence.org search strategy [14], a set of relatively powerful search filters was created. These search methods are outlined as follows:

Inclusion criteria for identifying systematic reviews

As recommended by Mahlungulu et al. (2007) and Lee et al. (2012), systematic reviews were included if they met the following criteria [14, 15]:

- The reviews covered the specific population of interest—patients with type 2 diabetes
- The reviews covered the intervention type—referral systems
- The reviews included outcomes for effective referral systems
- The reviews were systematic, published in English, and were medium to high quality as judged by the AMSTAR checklist.

Search strategy for identifying systematic reviews

The search included systematic reviews, regardless of the date they were conducted, using different databases in order to include the most suitable studies that would facilitate an understanding of what is current best practice. The following databases, identified with the assistance of the health librarian and the systematic reviews methods expert, were searched in May 2014: Health System Evidence, Cochrane Library, Embase, PubMed, and CINAHL.

Quality assessment of reviews

The Health Technologies Assessment robot database from the Canadian Agency for Drugs and Technology in Health (CADTH, 2014) (http://www.cadth.ca/en/resources/rx-for-change/database/) was utilised in order to conduct the quality assessment for this paper [12]. The Measurement Tool to Assess Systematic Reviews (AMSTAR) was employed for the assessment of the systematic reviews. AMSTAR has resulted in a growth in the number of systematic reviews for evidence-based health care [16]. This growth has led to certain benefits and also some risks. The benefits are that the decisions of researchers can be based on accurate, reliable, easily understood and detailed synopses of the evidence that is available. This causes a minimum amount of errors and biases being introduced in the work. On the other hand, variable quality and the absence of empirical validation are some of the risks of systematic reviews [16].

In this paper, AMSTAR was used to assess the included systematic reviews, as it is a tool with good inter-observer agreement, test-retest reliability, and good face and construct validity [16]. It is simple in its usage and it has previously been positively evaluated in several papers [16]. Also, AMSTAR has greatly improved the assessment of the standard of the methods applied for systematic reviews. Furthermore, CADTH (2014) supports it, and it has been cited over 200 times in the past 3 years [12]. It is a very practical tool as it does not take very long to complete, and the final decision is reached easily because the guidelines are easy to understand. AMSTAR has exhibited a reasonable degree of reliability and construct validity.

Results

Systematic review search

The search for systematic reviews found 47 article citations from five databases; 19 from the Health Systems Evidence, 15 from the Cochrane Library, six from Embase. seven from PubMed and 0 from the CINAHL database; two duplicates were removed leaving a total of 45 reviews. After screening the title and abstracts for relevance to the research question, seven articles remained. After this, a full document review was undertaken and three articles were identified as relevant. As previously stated, these included systematic reviews checked for their AMSTAR quality using CADTH (2014) [12]. Two of the three were Cochrane Reviews [17, 18]. The third systematic review was published in the British Journal of General Practice by Faulkner et al. (2003) [19]. Figure 2 shows the stages of retrieving systematic reviews using PRISMA.

Quality assessment of systematic reviews using CADTH

The included systematic reviews were assessed for quality using the Health Technologies Assessment database on the CADTH (2014) robot web site [12]. The included systematic reviews were rated as medium or high quality. Akbari et al. (2005) is a medium quality review, with a score of 7 out of 11 [12]. It was difficult to establish

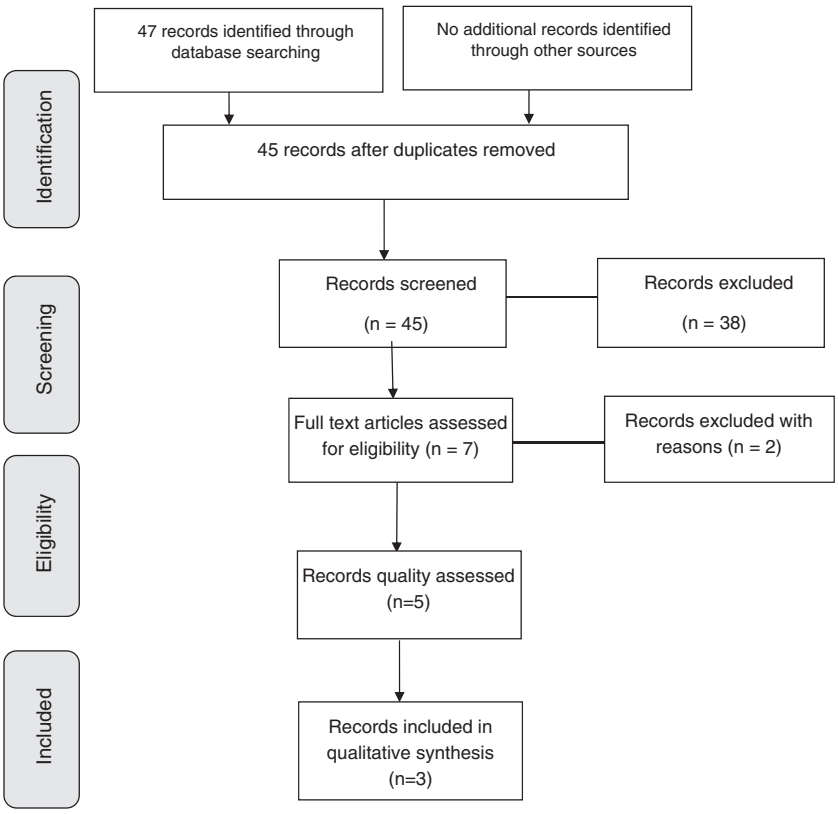

Fig. 2 PRISMA flow diagram of the systematic reviews. Source: Moher et al. (2009). doi:10.1371/journal.pmed.1000097.g001 [20]

conclusions on the associated outcomes due to the limited number of studies identified [12]. Too few studies were identified to draw conclusions on other related outcomes. Faulkner et al. (2003) is a high quality review, with a score of 8 out of 11 [12]. It is challenging to make observations about the efficacy of sharing educational materials, meetings, and multifaceted interventions on determining related outcomes as only a few studies have been identified. Renders et al. (2000) was also a medium quality review with a score of 7 out of 11 [12].

Description of the systematic reviews included in the study

The number of included primary studies in the systematic reviews totalled 80. Only systematic reviews published in English and that reported on best practice for referral systems were included. This includes randomised controlled trials (RCTs), controlled clinical trials (CCTs), controlled before and after studies (CBAs), interrupted time series (ITSs), observational controlled studies, and economic appraisal and reports [12]. The intervention categories were divided into educational, organisational, financial, professional, patient/public, and patient-oriented interventions (as shown in Table 2). Combining two or more interventions is significant for better referral systems (Faulkner et al. 2003) [19]. Table 2 summarises the included systematic reviews.

Analysis of systematic reviews using PIO

Table 3 provides an analysis of the systematic reviews using PIO components. The included reviews fit reasonably well with the review questions of interest in this paper.

Summary of effective referral interventions from systematic reviews

After analysing the three systematic reviews, quality referral interventions were identified that were considered as being effective in the management of diabetes and other chronic diseases. These interventions included the circulation of protocols, structured referral letters and a centralised computerised system. Some other referral interventions such as the remuneration system, fund holding scheme, and granting an introduction to a private specialist, or a specialist who is hospital-based at the same cost to the patient were effective only in terms of the quantity of referrals. Table 4 summarises the findings on referral interventions.

Development of Ten EBP factors

Ten referral factors were identified from the literature as being effective. Factors 1–6 are based on the US National Diabetic Education Program (NDEP) and adapted from Corrigan, Donaldson, Kohn, Maguire, and Pike (2001) [21]. They

Table 2 Characteristics of included systematic reviews

Year	Study author	Setting	No. of included articles	Quality rating	Findings about referral systems
2005	Akbari et al. [17]	All primary care practices such as—hospitals, outpatient clinics; public and private ownership—unspecified/unclear; academic—unspecified/unclear	17	7/11	There were nine studies appraised of professional educational intercessions (these included 14 correlations), four studies appraised organisational intercessions and four investigated financial intercessions (five correlations included). Effective education: If guidelines were disseminated using structured referral letters and teaching about referral by health care professionals Organisation: Getting a second opinion before referring or reinforcing services that are provided before issuing a referral (little evidence) Financial: Remuneration system, fund holding program, and granting access to a private specialist or a specialist based at a hospital costing the same to the patient Effective on quantity of referrals Not effective Education: If guidelines are distributed and health care professionals provided with good feedback as to how they are referring Organisation: Na Financial: Remuneration system, fund holding program, and granting access to a private specialist or a specialist based at a hospital costing the same to the patient Not effective on the quality of referral
2003	Faulkner et al. [19]	Primary care practices, outpatient clinics, communities, ownership—not specified, academic—not specified	44	8/11	Effective professional (n = 16). Education or guidelines Organisational (n = 22 General practice in-house primary healthcare team and specialist General practitioner fundholding open-access referral schemes Financial/regulatory n = 6 studies in 7 reports Fundholding remuneration Cost containment policy (The procedure of keeping control of the expenses needed for an organisation to operate or execute a project within pre-determined budgetary allowances The cost containment procedure is a crucial executive operation that helps to keep monetary outlays down to only imperative and calculated expenses so as to satisfy economic objectives). Subsidies referral to private care (effective on quantity) Patient/public: Information campaigns: Not effective: na
2000	Renders et al. [18]	Primary care practices, hospitals, ownership—not specified, academic—teaching, academic—Not specified	41	7/11	Effective professional: Multifaceted professional intervention organisational: Centrally computerised recording system or patients who are routinely in contact with nurses Patient-oriented intervention through nurses: Not effective: na

suggest six important factors related to the effective coordination of successful referrals and transitions, namely that a referral should be timely, safe, effective, patient-centred, efficient, and equitable [21]. Timely referral means that a patients' referral transitions to a consultation as promptly as possible [21]. This is also in agreement with the WHO referral system guidelines [4]. The needs of patients are more likely to be met when health care practices have staff who are committed, therefore, referrals and transitions have a better success rate [4], which also facilitates the transfer of clinical information. Secondly, safe referrals are planned and managed to prevent the destruction of patient referral information due to medical or administrative errors [21]. To avoid affecting a patient's health due to mislaid information, the referral system should convey medical and administrative information. The third factor is for referrals to be effective, which means that referrals are based on scientific knowledge, and executed well to maximise their benefit [21]. If there is not enough information in the referral letter, this could have an undesirable outcome for the patient and the secondary healthcare provider alike. The

Table 3 Analysis of the systematic reviews using PIO

PIO components	Akbari et al. [17]	Faulkner et al. [19]	Renders et al. [18]
Population	Primary care physicians Specialist physicians working in community outpatient settings or hospitals	Patients Primary care Secondary care	Patients with type 2 diabetes
Intervention	Professional education: (n = 9) Guidelines disseminated with structured referral letters and involvement of health care professionals who are consultants in teaching about referral systems. Guidelines distributed and health care professionals provided with feedback. Organisational: Second view before referring, or improving the health facilities provided before a referral. (little evidence) Financial: Remuneration system, fund holding program; Provision of access to private specialist, or a hospital based specialist at the same cost to the patient. Effective on quantity of referrals.	Professional: (n = 16). Education or guidelines Organisational (n = 22 General practice in-house primary healthcare team and specialist. General practitioner fundholding Open-access referral schemes Financial/regulatory: n = 6 studies in 7 reports Fundholding Remuneration Cost containment policy Subsidies referral to private care. Patient/public: Information campaigns	Professional: (n = 12) Multifaceted professional intervention Organisational: (n = 9) Central computerised tracking system Or nurses who regularly contact the patient. Professional plus Organisational: (n = 20) Patient-oriented (n = 15) Intervention through nurses.
Outcome	17 studies were considered. Unsuccessful strategies: passive distribution of local referral procedures, feedback of referral rates and conversations with an autonomous medical adviser. Successful strategies: distributing procedures; structured referral sheets; participation of advisers in education. Organisation interventions: successful in 4 studies. Included patient supervision by family physicians, physiotherapist's link to general procedures, a slot system for referrals, & second opinion before referral. Financial interventions: 4 studies. Decrease in referral rates when shifted from co-payment fee (capitation) system to mixed capitation & fee-for-service system; also from a fee-for-service system to a capitation- based system. Private professionals: An increase in the percentage of patients referred to specialised services; referral rates were not affected as a whole. General practice fund-holding scheme in the UK: decline in referral rates.	34 articles, 10 more studies included in an updated search. 2 studies included an economic analysis. In most of the studies, referral was not the main result obtained. Referral rates were usually affected by professional interventions. Little impact of experts' outreach or other primary care-based specialised provider schemes on referral rates. Financial interventions aimed to shift referrals from primary to specialised secondary care. Economic aspects of 14 studies were of poor quality. There was no pattern of variation in the referral costs or in total costs when the grouping occurring by the kind of intervention.	The intervention technique was versatile. Results were enhanced when a mix of professional interventions were used. The impact on patient outcomes was rarely evaluated. Positive impact on process outcomes was observed when there were preparations for follow- up (organisational intervention). Positive impact on patients' health outcomes was also observed in multiple interventions where the patient education was incorporated or where the duty of nurse was improved.

Table 4 Findings from systematic review

Referral factors	Effective	Not effective
Akbari et al. 2005 [17]	Education: Guidelines that are circulated with the structured referral letters, and teaching about the referral by health care professionals Organisation: Second view before referral, or improving the health services provided before a referral (little evidence) Financial: Remuneration system, fundholding scheme, and providing access to private specialist or a specialist who is based at a hospital, at the same cost to the patient. Effective on quantity of referrals.	Education: If guidelines are distributed and health care professionals are provided with feedback on how they are referring Organisation: Not stated Financial: Remuneration system, fundholding scheme, and providing access to private specialists or specialists who are based at a hospital, at the same cost to the patient. Not effective on the quality of referral.
Faulkner et al. 2003 [19]	Professional: Education or guidelines Organisational: General practice in-house primary healthcare team and specialist General practitioner fundholding Open-access referral schemes Financial/regulatory: Fund holding remuneration Cost containment policy subsidies referral to private care (effective on quantity). Patient/public: Information campaigns	Not stated
Renders et al. 2000 [18]	Professional: Multifaceted professional intervention. Organisational: Centrally computerised recording system or patients who are routinely checked by nurses. Patient-oriented intervention through nurses.	Not stated

fourth factor is that referral services should be patient-centred and responsive to patient and family needs and preferences [21]. Referrals are more likely to be successful if the providers and consultants who are referring the patients are well aware of the other's expectations and inclinations [17, 19]. In addition, the health practices that are referring patients should have appropriate staff and information procedures to enable them to assist patients and to ensure that patients are directed to where they need to go, based on their medical need. The involvement of patients and family alike is not only beneficial and supportive; it can help the referral process by keeping them well informed [4]. Patients and families can play a significant role in the referral process when a patient-centred approach is used by the referral coordinator or the physician.

The fifth factor for referrals to be efficient is that referrals should be confined to health care providers who are most likely to be of benefit to patients, and therefore avoid the need for patients to double up on services [21]. The sixth factor is that referrals should be equitable. This means that the accessibility and standard of referrals does not vary as a result of the

personal characteristics of patients [21]. The first priority of the WHO components of the referral system summary is availability and quality of care [4].

From the systematic reviews on the best practice in relation to referrals, three important factors were identified.

The seventh factor is that a referral letter sheet should be structured [17]. WHO also stated that a referral form should be standardised throughout the network of health services to ensure that the same essential information is provided whenever a referral is initiated [4]. The eighth important factor is to disseminate referral guidelines on primary and secondary health care [17]. The ninth factor is using central computerised systems in the referral process [18].

The tenth factor is based on a study conducted in Denmark on referrals in relation to type 2 diabetes, suggesting that referral to secondary health care should have inclusion criteria to avoid the inclusion of many new unnecessary cases [22]. Therefore, the last factor is that every referral to secondary health care should contain inclusion criteria for every referred patient, as this is significant in managing the referral system. When developed, this tool will be used to gather information on the most important factors for referrals system in any setting and between all levels of care. As previously noted, these criteria of good referral services are based on several sources, namely the systematic reviews by Corrigan et al. (2001), the WHO (2014), Akbari et al. (2005), and Renders et al. (2000), and studies on referral systems and how they should be utilised in different settings [4, 17, 18, 21, 22].

Discussion

This paper aimed to develop a referral tool that is EBP. The tool was based on ten important factors that could be used to assess referral systems in any context. The ten factors identified that an EBP referral system should be safe, timely, effective, efficient, patient-centred, and equitable (Corrigan et al. 2001; WHO, 2014); a referral letter should be structured (Akbari et al. 2005); referral letter guidelines should be disseminated (Akbari et al. 2005; Faulkner et al. 2003), a central computerised system should be used (Herrin et al. 2012; Renders et al. 2000), and inclusion criteria of referred patients should be given in a referral letter (Hansen et al. 2014) [4, 17, 18, 21, 22]. Examining one factor or combination of factors of referrals will lead to better healthcare systems and, in turn, better health care quality for patients. The demand for health services has increased rapidly in recent years in both developed and developing countries which have brought about rapid change to the structure of healthcare system. In order to overcome the issues of high demand of referral and its low quality, this tool will be helpful for health practitioners as it has six domains of healthcare quality as well as four important factors that are specifically related to referral. To enhance the efficiency of primary care, referral with high quality could avoid the repeated health services for the patient at secondary care which in turn will make the referral process more efficient and cost-effective. This tool also will contribute to high quality direct care to patients in timely manner.

Conclusion

This ten-factor assessment tool could be used to assess referral systems as it uses EBP on high quality studies and organisation. Using this tool could help researchers of referral systems to assess the problems of the referral systems in their practice and address these problems in light of the ten factors. The study is limited in that there are not many systematic reviews that investigate referral systems. However, the inclusion of new studies and the use of quality standards on referrals overcome the paucity of systematic reviews.

Compliance with ethical standards

Funding This study was funded by Madarek for Health Consultation. (Grant number: IRF-001).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):124–128
DOI 10.1007/s13410-017-0549-2

SHORT ARTICLE

Type 2 diabetes mellitus remission after bariatric surgery in Hispanic patients from Costa Rica

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Received: 19 September 2016 / Accepted: 7 January 2017 / Published online: 13 January 2017

Abstract Although bariatric surgery is considered a cost-effective technique to treat type 2 diabetes mellitus (T2DM), long-term clinical outcomes of this surgical procedure are unknown in our region. The aim of this study was to report the T2DM remission rate at 3 years in Hispanic patients treated at our referral center. We retrospectively reviewed clinical records from all T2DM patients who underwent bariatric surgery in our center. T2DM remission rate at 3 years was considered if patients had A1c ≤ 5.7% and fasting glucose ≤100 mg/dl in the absence of diabetic medication. Univariate and multivariate analyses were performed to detect prognostic variables of remission. Thirty-three patients were included in this study. Nineteen subjects underwent gastric sleeve and 14 patients Roux-en-Y gastric bypass (RYGB). Mean body mass index at baseline was 51.1 ± 8.8 kg/m^2 and mean glycosylated hemoglobin was 7.8 ± 1.8%. After 3 years, the percentage of weight change from baseline was 26.7% ± 10%. T2DM remission was achieved by 22 patients (66.6%). T2DM remission did not vary according to the type of surgery. Nevertheless, patients with T2DM remission had shorter duration of known diabetes (2.0 years vs. 5.5 years; P = 0.047) and larger weight change loss (−29.6% vs. −22.5%; P = 0.04) than patients without remission. After multivariate analysis, only fasting glucose plasma levels were associated with T2DM remission. Bariatric surgery is associated with high T2DM remission rate among Hispanic patients from Costa Rica. Prospective data are needed to determine predictors of T2DM remission.

Keywords Bariatric surgery · Costa Rica · Diabetes mellitus · Hispanic Americans · Obesity

Introduction

The prevalence of obesity and type 2 diabetes mellitus (T2DM) is increasing worldwide, especially in unindustrialized regions [1]. The high burden of this disease in developing countries is partly caused by the lack of effective measures to prevent the epidemics of overweight and obesity. In our country, the reported prevalence of T2DM was 8.6% in 2010, and the prevalence of overweight and obesity was 77.3 and 59.7% in men and women, respectively [2]. Similarly, the global health expenditure on diabetes is expected to total USD 490 billion in 2030, and the estimated prevalence of T2DM will increase by 69% in some unindustrialized areas [3].

Bariatric surgery is a procedure that is performed on adult patients with a body mass index (BMI) of ≥40 kg/m^2 without comorbid illness and adults with a BMI of 35.0 to 39.9 kg/m^2 with one serious comorbidity (such as T2DM) [4]. Bariatric procedures include Roux-en-Y gastric bypass (RYGB), adjustable gastric banding, biliopancreatic diversion, duodenal switch, and sleeve gastrectomy. These techniques have been recently incorporated by the Costa Rica Social Security System with unknown results. Although 78% of T2DM patients exhibit diabetes remission after bariatric surgery [5], few studies have evaluated the role of this surgical procedure in Hispanic patients. These patients have different access to medical care and different ethnic background than the original population for which the benefit of these techniques was proven [6–8]. Therefore, the aim of this study was to report the

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T2DM remission rate at 3 years in our center and to provide "real-world" evidence for this procedure among T2DM Hispanic patients.

Materials and methods

Universe and sample size

This was a retrospective observational cohort study with data from the San Juan de Dios Hospital, a tertiary referral center located in San José, Costa Rica. We included only T2DM patients who underwent bariatric surgery during 2012. All patients were followed for 3 years after surgery. We excluded patients lost to follow up because no assumptions could be made about these data. We identified 110 consecutive patients who underwent bariatric surgery during the study period. Of them, only 33 patients met the inclusion criteria. The study was approved by the institutional ethical committee. For this type of study, formal consent was not required.

Data collection

We collected demographic and clinical variables from medical records. The following variables were recorded 1 month before and after the bariatric procedure (at months 3, 6, 12, 24, and 36): body weight, body mass index (BMI), blood pressure, fasting glucose level, glycosylated hemoglobin (A1c), total serum cholesterol, low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), serum triglycerides, and medications for diabetes control. Patients were followed up by a team of an endocrinologist, a general surgeon, a clinical nutritionist, and psychologist. A psychiatrist followed up all patients before and after surgery. All patients received preoperative assessment. The surgical procedure was carried out by the same surgery group and consisted of laparoscopic Roux-en-Y gastric bypass (RYGB) or laparoscopic sleeve gastrectomy, according to the surgeon's preference. The surgical procedures were performed following previously described techniques [9].

The primary outcome of this study was complete diabetes remission at 3 years, defined as A1c ≤5.7% and fasting glucose ≤100 mg/dl in the absence of diabetic medication [10]. Secondary outcomes included changes in weight, BMI, A1c, LDL, HDL cholesterol, serum triglycerides, and systolic and diastolic blood pressure. Diabetes was considered improved if patients still required oral medication at lower dosages than at baseline (but no insulin) and had A1c <7.0% [10]. Diabetes medications were titrated with dosage decreased if fasting and postprandial glucose levels were <120 and <160 mg/dL, respectively. Diabetes medications were discontinued if A1c levels remained <6.4%.

Statistical analysis

Categorical variables are presented as percentages and continuous variables are presented as means and standard deviations. Continuous variables before and after the surgical procedure were compared using the Friedman's test for repeated measures. The Fisher's exact test (for categorical variables) or the Mann–Whitney test (for continuous variables) were used to analyze differences between patients achieving T2DM remission and patients without remission. A multivariate logistic regression analysis was done to determine the association between baseline clinical variables and the probability of achieving T2DM remission as categorical variable. Only predictors significantly associated with remission in the univariate analysis with P values less than 0.10 were included in the multivariate mode by a stepwise method. A P value of less than 0.05 was considered statistically significant. Data were analyzed with SPSS 20.0 for Mac (IBM Corp., Chicago, Ill.).

Results

Table 1 describes clinical characteristics at baseline and during follow-up. Mean age at baseline was 42.4 ± 8.3 years. Twenty-three patients (70%) were female. The bariatric procedure performed was a gastric sleeve in 19 patients (57.6%) and a RYGB in the remaining 14 patients (43.4%). Mean diabetes mellitus duration was 4.4 ± 3.5 years before the surgical procedure. The percentage of weight change from baseline to follow up is shown in Fig. 1: −28.2 ± 9.0% at year 1, −29.0 ± 9.1% at year 2, and −26.7% ± 10% at year 3. There was a non-significant trend towards a higher percentage of weight loss at year 3 after the surgical procedure in patients who underwent RYGB vs. patients who underwent gastric sleeve (−30.5 ± 5.7% vs. −23.5 ± 12%, respectively; $P = 0.063$).

Figure 2 shows the evolution of A1c during follow-up. Three years after surgery, all patients had A1c values less than 7.0%. Diabetes mellitus remission at 3 years was achieved in 22 patients (66.6%) while the remaining 11 patients (33.3%) continued with diabetic medication. Nevertheless, there was a reduction in the number of oral anti-diabetic drugs in the latter group, and none of these patients continued with subcutaneous insulin. Therefore, all patients in this subgroup met the criteria for T2DM improvement. The percentage of patients receiving two or more anti-diabetic drugs before surgery was 48.5 vs. 9.1% after the surgical procedure ($P = 0.032$). T2DM remission rate did not vary according to the surgical procedure performed (gastric sleeve = 68.4% vs. RYGB = 71.4%; $P = 0.43$). However, patients who achieved complete T2DM remission had a shorter duration of known diabetes (2 years vs. 5.5 years; $P = 0.047$) and larger weight change loss at 2 and 3 years (−32.6% vs. −24.2%; $P = 0.04$ at 2 years and

Table 1 Clinical characteristics of the studied population at baseline and during follow-up

Variable	Baseline	3 moths	6 months	12 months	24 months	36 months	*P* value
Weight (kg)	135 ± 28.8	112 ± 23.8	99.5 ± 19.6	96.9 ± 18.1	92.7 ± 17.0	97.4 ± 18.6	<0.001*
BMI (kg/m^2)	51.1 ± 8.8	42.6 ± 8.1	37.8 ± 6.76	35.8 ± 6.2	34.8 ± 4.6	36.9 ± 5.7	<0.001*
Fasting plasma glucose (mg/dl)	152.0 ± 64.5	101 ± 18.3	99.1 ± 18.6	92.2 ± 19.1	92.8 ± 18.0	95.8 ± 36.6	0.004*
A1c (%)	7.8 ± 1.8	6.4 ± 1.3	5.8 ± 0.9	5.7 ± 0.4	5.6 ± 0.6	5.7 ± 0.6	0.018*
Serum cholesterol (mg/dl)	176.4 ± 38.8	169.0 ± 32.7	176.2 ± 40.7	174.4 ± 29.4	185.3 ± 36.3	180.1 ± 31.6	0.873
LDL cholesterol (mg/dl)	106.1 ± 31.3	102.2 ± 33.5	110.3 ± 38.0	104.0 ± 25.7	103.6 ± 38.5	111.9 ± 27.9	0.812
HDL cholesterol (mg/dl)	39.1 ± 10.4	35.3 ± 12.6	39.9 ± 9.6	48.4 ± 13.6	55.0 ± 12.6	48.3 ± 11.6	0.038*
Serum triglycerides (mg/dl)	179.6 ± 85.9	151.3 ± 60.2	129.1 ± 49.2	102.7 ± 41.3	129.1 ± 78.8	113.2 ± 49.7	0.588
SBP (mmHg)	125 ± 14.7	122.0 ± 10.9	135.0 ± 23.8	124.0 ± 13.5	128.2 ± 5.5	122.8 ± 15.3	0.232
DBP (mmHg)	76.1 ± 9.2	74.1 ± 8.9	83.7 ± 12.5	73.2 ± 11.6	71.2 ± 10.8	71.7 ± 8.1	0.544

BMI body mass index, *DBP* diastolic blood pressure, *A1c*: glycosylated hemoglobin, *HDL* high-density lipoprotein cholesterol, *LDL* low-density lipoprotein cholesterol, *SBP* systolic blood pressure

*Statistically significant at $P < 0.05$

−29.6% vs. −22.5%; $P = 0.04$ at 3 years) than patients who did not achieve T2DM remission 3 years after surgery. Table 2 resumes other baseline clinical variables and their association with T2DM remission. After multivariate logistic regression analysis, only baseline fasting plasma glucose levels was independently associated with the odds of not achieving remission; however, the magnitude of this relationship was small (Odds ratio 0.97; IC 95% 0.96–0.99; $P = 0.045$).

Discussion

There are few data reporting metabolic outcomes after bariatric surgery in patients with T2DM from Hispanic populations. Although previous studies have consistently shown the efficacy of this technique to induce T2DM remission [7–9], the ethnic background and access to medical care in our patients is different from the reported by these clinical trials. Indeed, recent studies have shown disparities in response to obesity treatments across patient groups, with scarce data regarding Latin–American populations [11]. Although previous reports have shown similar outcomes in Hispanic obese patients compared to non-Hispanic white subjects, the majority of these trials have been carried on in industrialized countries, disregarding social, biological, and behavioral factors that can affect weight loss and treatment adherence after bariatric surgery [12–14]. In contrast to the aforementioned studies, other authors report different weight loss and outcomes among Hispanic patients. For example, Cheung and colleagues showed a lower percentage of weight loss in Hispanic patients than in Caucasian counterparts [15]. Analogously, metabolic syndrome resolution has been reported to be lower in Hispanic patients than in Non-Hispanic white subjects [16].

Our results are in accordance with previous reports [5, 7–9, 12–14, 17, 18] since we showed a high proportion of patients achieving T2DM complete remission after 3 years of bariatric

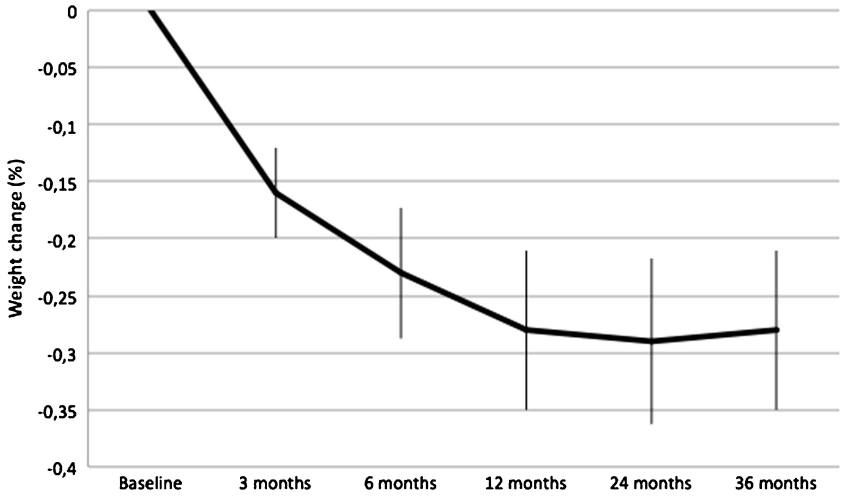

Fig. 1 Percentage of weight change from baseline to 36 months (*P* for trend <0.001)

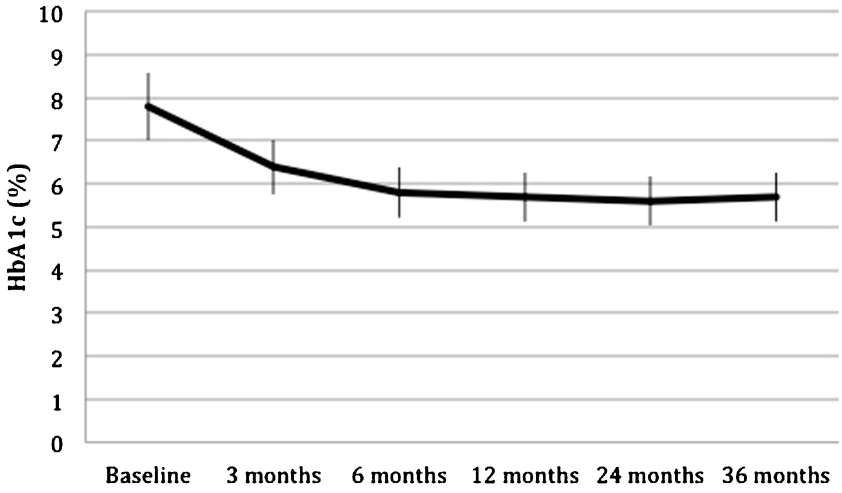

Fig. 2 A1c levels at baseline and during follow-up after bariatric surgery (*P* for trend = 0.018)

surgery. However, the magnitude of this remission is higher than the reported previously in the same time frame (ranging from 38 [17] to 40% [18]). Although we cannot exclude a type II error due to our small sample size, this finding must be corroborated by prospective data from our region.

At 3 years after the procedure, the observed mean percentage of weight loss (−26.7%) was similar to the described by Courculas and colleagues that reported a −25% weight change among patients who received RYGB [18]. In contrast to these and other authors [19], we did not find any significant difference between the surgical procedure performed in our cohort and the rate of T2DM remission. It has been postulated that after bariatric surgery, there is a benefit on glycemic control independent of weight loss due to an enhanced postprandial insulin secretion induced by several incretins such as glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) as well as changes in gut microbiota and bile acid composition that may differ between these two procedures [20]. Indeed, previous studies have demonstrated that RYGB confers a more durable effect on T2DM compared to gastric sleeve and a tendency for lower relapses even the similar weight loss achieves for both techniques [21]. Nevertheless, differences in definitions and follow-up periods can explain the aforementioned discrepancies among studies.

Our findings showed that patients with the high weight loss at 3 years, low fasting glycemia before surgery, and short diabetes duration were more likely to have T2DM remission. These results are in accordance with previous reports [22, 23]; however, they have been carried out in non-Hispanic patients with some conflicted data regarding the identification of prognostic variables.

Our study has some limitations due to its single-center design, small sample size, and retrospective design. Furthermore, although all patients in our cohort were followed-up for 3 years after bariatric surgery, we cannot exclude the presence of confounding variables that could bias our findings. Despite these caveats, we demonstrate for the first time a relatively high T2DM remission rate after bariatric surgery among Hispanic patients from a Latin–American country. The retrospective design of this study warrants further confirmation in a prospective cohort.

Table 2 Association between variables at baseline and the probability of achieving complete type 2 diabetes mellitus remission at 3 years after bariatric surgery

Variable	Patients without T2DM remission (*n* = 11; 33.3%)	Patients with T2DM remission (*n* = 22; 66.6%)	*P* value (univariate analysis)	*P* value (multivariate analyses)
Age (years)	44.1 ± 8.3	41.6 ± 8.7	0.44	
Weight (Kg)	126.0 ± 30.8	139.5 ± 28.3	0.27	
BMI (Kg/-m^2)	46.9 ± 7.6	52.9 ± 9.0	0.06	0.307
Fasting plasma glucose (mg/-dl)	203.4 ± 91.1	130.2 ± 31.3	0.02*	0.045*
A1c (%)	8.6 ± 2.4	7.4 ± 1.4	0.24	

BMI body mass index, *A1c* glycosylated hemoglobin

*Statistically significant at $P < 0.05$

Authors' contributions Dr. C. Chen-Ku and Dr. M. Alfaro were involved in the conception and design. Dr. M. Alfaro contributed in the acquisition of data. Dr. A. Ramos-Esquivel contributed in the analysis and interpretation of data. Dr. C. Chen-Ku, Dr. M. Alfaro and Dr. A. Ramos-Esquivel contributed in the analysis of data and were involved in drafting the article. All the authors revised the

article for important intellectual content and approved the final version of this manuscript.

Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The protocol of this study was approved for the Bioethics Institutional Committee.

Conflicts of interest The authors declare that they have no conflict of interest.

Informed consent For this type of study, informed consent was not needed as only anonymized patient information was used.

Financial support None.

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Int J Diabetes Dev Ctries (January–March 2018) 38(1):129–130
DOI 10.1007/s13410-017-0569-y

LETTER TO THE EDITOR

Insulin-induced thirst: are we overlooking it?

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Received: 31 May 2017 / Accepted: 19 June 2017 / Published online: 7 July 2017

Time and again, we have been overwhelmed by little pearls of medicine which we gather along the way, in a journey to understand the functioning of human body in health and disease. In recent times, we came across two females with long-standing uncontrolled type 2 diabetes (HbA1c of more than 12%), who complained of an increased perception of thirst following sub-cutaneous insulin administration. They experienced thirst soon after initiating insulin that lasted for about 2–3 weeks. The response developed after about 30–60 min of each insulin shot. Capillary and plasma glucose values measured during thirst were between 120 and 150 mg/dl. Oblivious of the physiological effects of insulin on body water homeostasis, we had ignored the observation initially. The first lady even lost to follow-up as we disregarded her complaints repeatedly and attributed it to a psychiatric issue.

The underlying mechanism of this dipsogenic response to insulin remains largely unknown. A study, done long back on a small group of human volunteers revealed significantly higher water intake after intravenous insulin injections when compared to saline. It was also observed that onset of water intake was concurrent with maximal dip in plasma glucose levels following intravenous insulin. In addition, plasma renin activity (PRA) and hematocrit levels were found to be significantly higher in the insulin-treated group when compared to basal conditions or after saline injections [1]. The effects of insulin-induced hypoglycemia on PRA were also assessed in subsets of normal human volunteers, adrenalectomised patients and patients with hypopituitarism. PRA, found to be significantly raised in all three groups, was amenable to blockade by propranolol [2]. A study assessing the role of glucose in insulin-induced drinking responses observed that the maximal dipsogenic response occurred during a rapid decline in blood glucose concentration which could be abolished by gastric administration of glucose. Thus, it appears that insulin-induced hypoglycemia results in a central sympathetic reflex stimulation of renin which leads to production of angiotensin II, a potent dipsogen. In all likelihood, this response is neither adrenal nor pituitary dependent, nor is the peripheral renin angiotensin system involved. Central angiotensin II, possibly, is the most important dipsogenic mediator underlying insulin-induced drinking response [3]. None of our patients had documented hypoglycemia during the thirst response. However, uncontrolled hyperglycemia for prolonged period is known to produce a right-ward shift of the set points of counter-regulatory hormone secretion and neuronal mechanisms of hypoglycemic response, a phenomenon known as relative hypoglycemia.

Even more interesting is the fact that there seems to be a sex-specific difference in insulin-induced drinking, suggesting a role of gonadal steroids in neuronal structures or

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mechanisms underlying drinking behaviour that is established before puberty [4]. Estrogen seems to have a stimulating effect on insulin-induced thirst irrespective of the genotypic sex. Both of our patients being female are perhaps another testimony to this hypothesis.

Lastly, psychological responses indicated that insulin elicits thirst sensation prior to hunger [5]. Endogenous insulin thus plays a possible role in meal-related thirst. Further, large-scale studies can reflect and yield a better understanding of such unusual and interesting side effect of insulin therapy.

Authors' contributions PPC and SNB were involved in management of the patients, literature search and preparing the draft.

SC was involved in management of the patient, providing intellectual inputs and finalizing the draft.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Role of funding source None.

Ethics committee approval This is not a study. In this article, we share some of our clinical experiences. Ethics committee approval is not required for this article. Institutional ethics committee approves case studies.

Consent The article does not contain any figures revealing the identification of any patient in any form.

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VISION STATEMENT

To be recognized as a global leader for clinical care, education, training, research, advocacy and capacity building in the field of diabetes.

MISSION STATEMENT

1. Promotion of excellence in diabetes care to make India the Diabetes Care Capital
2. Empowerment of persons living with diabetes
3. Support for diabetes research
4. Dissemination of information and knowledge in diabetes care
5. Advocacy for the cause of diabetology

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- For providing research grants, RSSDI invites proposals from Indian scientists, interested in conducting original research in the field of diabetes mellitus. Furthermore, limited grants are also available for the students of medical colleges for smaller projects.
- There is no deadline for submission of the proposals, which can be sent throughout the year. These proposals may fall into one of the following three categories:

1. Projects involving funding up to Rs 40,000 per project (preference will be given to young scientists <40 years).
2. Projects involving funding up to 10 lakhs.
3. We also invite proposals for more than 10 Lakhs as major projects but these have to be preferably multicentric.

- The detailed proposals should include the following:

  ◊ Title, names of principal and co-investigators, summary, introduction/background, review of literature, aims, methodology, study design, and detailed plan of work and bibliography. Brief biodata of principal investigator and other co-investigators
  ◊ Importance of work in the context of national priorities. Detailed budget sought along with full justification/ proposed utilization, of funding sought from RSSDI
  ◊ Whether the project is being partly funded from any other source? If yes, please mention the source and the amount received.
  ◊ Ethical committee clearance of the institution or other bonafide body.

Travel grants for young diabetes researchers to attend International Conferences

Criteria's for the travel grant are as follows:

- Applicant should apply 2 months in advance.
- Travel Grant is open only to the RSSDI members.
- Applicant should submit Oral paper / Poster acceptance document to RSSDI Secretariat.
- Applicant should submit Declaration that he/she has not receiving grant from any other agency / Organization – In case of receiving grant from any other Organization, RSSDI shall pay only the exceeding amount not covered by that agency.

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(IN ASSOCIATION WITH JAIPUR NATIONAL UNIVERSITY)

Research Society for the Study of Diabetes in India (RSSDI) was founded by Prof. M.M.S. Ahuja in 1972. RSSDI is the largest body of professional doctors and researchers in Asia, working in the area of Diabetes & is the National Body recognized by IDF (International Diabetes Federation). One of the key areas of focus is to train doctors at all levels to better manage Diabetes and its complications. RSSDI recognizes this problem and runs a well-structured, full time, residential "Advanced Certificate Course in Diabetology". This two-year course is like any other post graduate course and has immensely helped doctors to practice better diabetes care. RSSDI has carefully looked into all aspects of this course & has accredited & recognized 16 centres across India at present and more centers are being inspected for accreditation. National Faculties and experts of RSSDI chosen from Academia visit these centers from time to time to ensure high standards. Now this Advanced Certificate Course has Dual Accreditation from RSSDI and Jaipur National University.

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2.	North Delhi Diabetes Centre	New Delhi, Delhi
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4.	Totall Diabetes Hormone Institute	Indore, Madhya Pradesh
5.	Dia Care A Complete Diabetes Care Centre	Ahemdabad, Gujarat
6.	Sonal Diabetes Hospital	Surat, Gujarat
7.	Jothydev's Diabetes and Research Center	Trivandrum, Kerala
8.	Advanced Endocrine & Diabetes Hospital	Hyderabad, Telangana
9.	G D Hospitals and Diabetes Institute	Kolkata, West Bengal
10.	Aditya Diagnostics & Hospital	Dibrugarh, Assam
11.	Sunil's Diabetes Care N' Research Centre Pvt Ltd.	Nagpur, Maharashtra
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Duration: 2 Years – Post MBBS & 1 Year - Post MD / DNB (Gen - Medicine)* (Full Time) Educational.

Qualification: A candidate must possess MBBS degree from ANY of the recognized university approved by Medical Council of India (*The duration of the course is 1 Year for those with MD/ DNB in Internal Medicine. Candidates having MD degree in other specialties will have to do the course over 2 Years).

Number of seats: 2 seats per year for every eligible teacher as per rules of Medical Council of India (MCI).

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Acknowledgement

We are grateful to the following reviewers for reviewing articles for International Journal in Diabetes in Developing Countries in the year 2017.

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International Journal of Diabetes in Developing Countries

Volume 38 | Issue 1 | January–March 2018

Springer